VOLUME 33 AUGUST 1955 NUMBER 4

Canadian Journal of Zoology

Editor: T. W. M. CAMERON

Associate Editors:

N. J. BERRILL, *McGill University*
I. MCT. COWAN, *University of British Columbia*
F. E. J. FRY, *University of Toronto*
F. R. HAYES, *Dalhousie University*
D. S. RAWSON, *University of Saskatchewan*
W. E. RICKER, *Pacific Biological Station, Nanaimo, B.C.*
J. L. TREMBLAY, *Laval University*
V. B. WIGGLESWORTH, *Cambridge University*

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF ZOOLOGY

(Formerly Section D, Canadian Journal of Research)

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF ZOOLOGY and six other journals devoted to the publication, in English or French, of the results of original scientific research. Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies.

EDITORIAL BOARD

Representatives of the National Research Council

A. N. CAMPBELL, *University of Manitoba*
G. E. HALL, *University of Western Ontario*
E. G. D. MURRAY, *McGill University*
D. L. THOMSON, *McGill University*
W. H. WATSON (Chairman), *University of Toronto*

Editors of the Journals

D. L. BAILEY, *University of Toronto*
T. W. M. CAMERON, *Macdonald College*
J. B. COLLIP, *University of Western Ontario*
G. A. LEDINGHAM, *National Research Council*
LÉO MARION, *National Research Council*
R. G. E. MURRAY, *University of Western Ontario*
G. M. VOLKOFF, *University of British Columbia*

Representatives of Societies

D. L. BAILEY, *University of Toronto*
Royal Society of Canada
T. W. M. CAMERON, *Macdonald College*
Royal Society of Canada
J. B. COLLIP, *University of Western Ontario*
Canadian Physiological Society
R. G. E. MURRAY, *University of Western Ontario*
Canadian Society of Microbiologists
H. G. THODE, *McMaster University*
Chemical Institute of Canada
T. THORVALDSON, *University of Saskatchewan*
Royal Society of Canada
G. M. VOLKOFF, *University of British Columbia*
Royal Society of Canada; Canadian Association of Physicists

Ex officio

LÉO MARION (Editor-in-Chief), *National Research Council*

Manuscripts for publication should be submitted to Dr. Léo Marion, Editor-in-Chief, Canadian Journal of Zoology, National Research Council, Ottawa 2, Canada.
(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover))

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration, National Research Council, Ottawa 2, Canada.

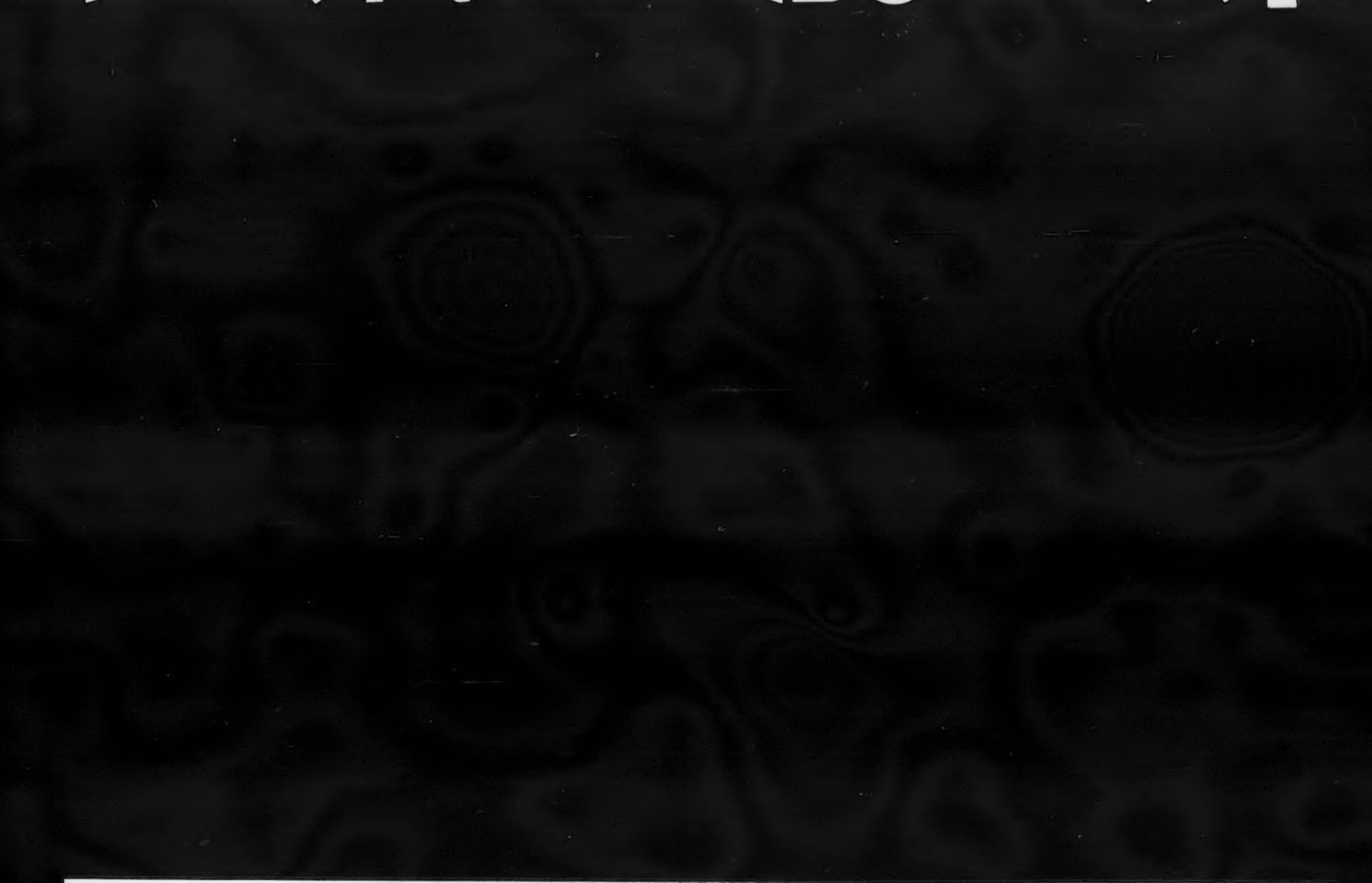
Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Bimonthly	$3.00 a year
Canadian Journal of Botany	Bimonthly	$4.00
Canadian Journal of Chemistry	Monthly	$5.00
Canadian Journal of Microbiology*	Bimonthly	$3.00
Canadian Journal of Physics	Monthly	$4.00
Canadian Journal of Technology	Bimonthly	$3.00
Canadian Journal of Zoology	Bimonthly	$3.00

The price of single numbers of all journals is 75 cents.

** Volume 1 will combine three numbers published in 1954 with six published in 1955 and will be available at the regular annual subscription rate of $3.00.*



Canadian Journal of Zoology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 33 AUGUST 1955 NUMBER 4

THE DEVELOPMENT OF SAMPLING TECHNIQUES FOR FOREST INSECT DEFOLIATORS, WITH PARTICULAR REFERENCE TO THE SPRUCE BUDWORM[1]

BY R. F. MORRIS[2]

Table of Contents

	PAGE
Abstract	226
1. Introduction	226
2. The expression of population	227
3. The objects of sampling	229
4. The timing of sampling	230
4.1 General considerations	230
4.2 The spruce budworm	231
4.3 Retention of egg masses on the foliage	234
4.4 Retention of pupal cases on the foliage	235
5. The mechanics of sampling	237
5.1 Collecting the sample branches	237
5.2 Examining the foliage	239
5.3 Checking the foliage	240
6. Selection of the universe	242
7. Selection of the sample unit	245
8. Representativeness in the tree	247
8.1 Distribution of budworm population	247
8.2 Distribution of branch surface	250
8.3 Representative sampling	251
9. Representativeness in the stand	253
9.1 Distribution of population in a two-storied stand	253
9.2 Selection of trees in each story	256
10. Correlation between successive samples	258
11. Transformation of data	261
12. Optimum sample size	268
12.1 Degree of precision required	269
12.2 Number of sample units per tree	271
12.3 Number of trees per plot	274
12.4 Number of clusters per plot	278
12.5 Influence of population level	279
12.6 The cost function	282
13. Absolute population	285
14. Incidental data	288
14.1 Population intensity	288
14.2 Life table data	289
14.3 Other sampling systems	290
15. Discussion	290
Acknowledgments	292
References	293

[1] *Manuscript received February 22, 1955.*
Contribution No. 209, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

[2] *Forest Biology Laboratory, Fredericton, New Brunswick.*

[The June number of this Journal (Can. J. Zoology, 33 : 107-223. 1955) was issued June 15, 1955.]

Abstract

The problems that arise in the development of sampling techniques are treated in sequence, the solutions illustrated being based on population studies on the spruce budworm in northwestern New Brunswick. Insect populations may be expressed in different ways, depending upon the objects of the sampling, and it is essential that these objects be carefully defined. In the spruce budworm studies the preparation of life tables is the primary objective, and population is expressed in terms of a basic unit (branch surface) and an absolute unit (the acre). The correct timing of sampling requires a knowledge of the insect's life history, and of the stability of the population in place and time. When insect signs (pupal cases, empty egg masses) are sampled, the retention factor gives rise to certain non-sampling errors. The mechanics of collecting foliage from tall trees is solved with the aid of aluminum pole pruners, extension ladders, tree trestles, and platforms. The mechanics of counting the insects by ocular examination of the foliage samples also gives rise to non-sampling errors, which can be minimized by adequate supervision and by check examination.

The universe for which each life table is prepared is a homogeneous forest stand. It is shown that a collection unit smaller than a whole branch, or its longitudinal one-half, is unlikely to be suitable for the measurement of absolute population. Intertree variance is the major source of population variance for the budworm, and for most other insects that have been studied intensively. Significant variance is also associated with crown levels, and the pattern of vertical distribution of the budworm is not predictable. The criterion of representativeness can be satisfied, however, by drawing samples from four crown levels in such a way that the intensity of sampling is equal in each level. The design found suitable for the budworm consists of *proportionate sampling* within the crown, *stratified sampling* by crown stories within the stand (with *sub-strata*, when necessary, according to host species or flowering condition), and *cluster sampling* within strata. Samples may be drawn from the same trees during successive budworm generations, and show correlations which appear to arise from the preferences of ovipositing adults. The effect of the correlations on sampling design and analysis is discussed.

The relation between mean and variance indicates that population data are represented satisfactorily by the negative binomial distribution; variance may be stabilized and additivity provided through the use of logarithmic transformation. Methods of calculating optimum sample size are illustrated for both the original and the transformed data. The cost function for the sampling design used in the budworm work is presented, and its use in the comparison of designs and the detection of limiting factors is demonstrated. The estimation of population per acre is achieved by means of regressions showing the relationships between foliage quantity, crown volume, and diameter of the trunk. Useful incidental data, including a measure of population intensity, and biological data for life tables, may be obtained during sampling. Also, the design can readily be modified to suit purposes of extensive insect survey or the biological assay of a single stimulus.

It is concluded that neither sampling nor non-sampling errors are likely to be prohibitive in forest insect population work. The relative magnitude of sampling errors varies inversely with the population mean, however, and the preparation of complete life tables may not be practical at endemic population levels. In any one generation of the insect it is generally necessary to accept error limits that seem large in relation to those of laboratory experimentation. But using the same sampling plots over a period of years, replication is achieved in time as well as in place, so the formation of erroneous conclusions on epidemiological phenomena seems improbable.

1. Introduction

This is the fourth in a series of papers (24, 23, 17) dealing with techniques for studing natural populations and mortality factors of the spruce budworm, *Choristoneura fumiferana* (Clem.). Although each paper is intended to be comprehensive in itself, a review of the introductory work on life tables (24) will be helpful in understanding the philosophy that underlay the development of the sampling techniques.

Population figures comprise the basic reference points in life tables, and it is important to compare life tables at different population levels. Also, certain mortality factors can be evaluated only by measuring population reductions at successive intervals in the life history. For these reasons, the sampling of natural populations in the forest has received much attention in the course of the present studies. This paper, like the others in the series, is restricted to the development of techniques. The results of the long-term study of population trends under different forest conditions will be reserved until the outbreak has run its course.

The steps in the development of a sampling technique may be conveniently represented as a series of problems or questions that arise, together with the relevant data and the proposed solution for each problem. The sections of the present paper present these problems in approximately the chronological order in which they would normally be encountered, starting with the basic question as to whether or not an intensive sampling program is necessary or justifiable. Actually, trial and error played a much larger part in the development of the techniques than might be supposed from this presentation. One reason for this was the lack of an adequate model for a similar defoliating insect. The title of the present paper, and the presentation of the various sampling problems, has therefore been made sufficiently broad to interest the investigator who may have similar sampling problems for other insects. The nature of the problems and the methods of solution should apply to other species and may help to obviate some of the less productive aspects of the trial and error method. The solutions themselves, on the other hand, will be applicable to only the spruce budworm, unless some of the distributional features are found to apply rather generally to other forest defoliators.

The work to be reported was carried out on the Green River Watershed in northwestern New Brunswick. The project was initiated in 1945, when the population of the spruce budworm was extremely low in this area. The first significant increase in population occurred in 1947 and the first mortality of overstory trees resulting from budworm defoliation was recorded in the fall of 1954. Although similar techniques are now being applied to the study of budworm populations on spruce, all results presented below apply to balsam fir, *Abies balsamea* (L.) Mill., which is the preferred host.

2. The Expression of Population

Forest insect populations may be expressed in various types of units, depending upon the objects of the work and method of sampling. Before outlining the development of the present method, it is proposed to classify the common types of population expression and suggest a terminology for them. The first four of these expressions generally result from *direct population studies*, that is, the insects themselves are found and counted. The fifth type of expression results from *indirect population studies* which involve, not the counting and recording of insect numbers, but the measurement of some factor that is known to be related to insect numbers.

1. Population intensity.—This is the expression of insect numbers in terms of the available food supply. In the case of the spruce budworm, for example, the population intensity would be the number of insects per bud or new shoot, or possibly the number of insects per unit weight of new foliage. The main food supply for the budworm is first the opening buds and later the needles of the new shoots. Population intensity is a useful expression, particularly if the main object of the study is to determine the number of insects associated with various degrees of damage to the host plant. It will be shown in a later section, however, that this expression often lacks stability if the main object is to study insect population trends over a period of years. Changes in the available food supply can affect population intensity drastically, even when no change in actual insect numbers has occurred.

2. Absolute population.—When population is measured in terms of a unit that cannot vary, we have an expression of absolute population. Sampling may be continued over an indefinite period of years with results that express changes only in actual insect population. For forest insects, the only unit that is strictly absolute is the unit of forest area, such as the acre. The quantity of insect food per tree and per acre, and the number and size of trees per acre, are dynamic units that vary from year to year, and usually in a definite upward or downward trend. Populations based on such units may therefore reflect *apparent* changes that are not accompanied by *absolute* changes in insect numbers. To illustrate with an extreme example, consider that only two trees are available for study on a given land area, and that one tree blows down at a stage when the insect population is motile. Concentration of the insects on the surviving tree results in a twofold increase in population per tree although there has been no real change in insect numbers.

3. Basic population.—Forest insects are distributed on the host trees, at least during certain important stages in the life history, where the direct measurement of their populations in terms of ground area is not feasible. Some basic unit for the sampling of population is therefore required and such a unit, with supporting data on the structure of the tree and the forest, can permit the conversion of results to absolute population and to population intensity. If there is no serious disturbance to the stand, basic population figures, without conversion, may be reasonably good indicators of absolute population changes over a limited period of years. The present paper will be concerned largely with a basic unit for the expression of population.

4. Relative population.—For some purposes it is sufficient to find gross, relative changes in population without expressing results in terms of any definite unit in the tree or the stand. A good example is the 'beating' method often used for purposes of forest insect survey. A canvas sheet of standard size is spread under one side of a tree and all branches over the sheet that can be reached with a 10-ft. pole are jarred to dislodge the insects. The proportion of the insects actually dislodged from the 'beaten' branches is unknown, as well as the proportion of the tree that has been sampled. The method is rapid and inexpensive, however, and provides useful information on the

species of insects present and their relative abundance from year to year. If the method is continued for many years in the same stand, true relativity may be lost because the development of the trees leaves a progressively smaller number of branches within the reach of the 10-ft. pole. Relative methods are usually suitable only to general insect surveys and will receive no further attention in the present paper.

5. Population indices.—For defoliating insects two common indices of population are the degree of defoliation of the host trees and the amount of frass-drop from the feeding larvae. In survey work defoliation may be estimated from the ground in broad percentage classes, and in more intensive studies it may be measured more accurately on sample branches. Defoliation is only a rough index of population and any serious attempt to relate the two accurately must take into account the changes in the annual foliage production of the tree resulting from repeated defoliation and from other factors. For certain types of defoliators, the writer (19) has shown that the frass-drop method can be used either for survey purposes or for more intensive studies where refinements permit the conversion of frass measurements to insect numbers. For the spruce budworm, the retention of a proportion of the frass in the feeding sites makes the technique unsuitable for intensive studies of larval population trend, but it has other uses which are discussed below (Sec. 9.2).

Population level and *population density* are so frequently used in denoting the general level of abundance that it seems unwise to associate them with any particular type of expression.

3. The Objects of Sampling

Population sampling of a forest insect may be conducted for one or more of a number of reasons. In general, the objects are concerned either with insect survey or with intensive studies. Extensive insect survey employs rapid, simple methods to determine the presence or absence of various species, to assess relative population, or to map an outbreak of a given species according to broad classes of infestation (23). Intensive studies are usually restricted to more or less permanent study areas or plots, selected to be representative of certain forest conditions. Their objects may range from a simple biological assay of one natural or applied stimulus to a complete study of all the complex factors involved in population changes.

These objects are so well known that they require no further discussion. However, the importance of clearly defining the objectives before any sampling program is initated cannot be emphasized too strongly. Any program initiated on the vague impulse of learning something about insect numbers is likely to be unproductive. The measurement of insect populations in the forest presents many difficult and unique problems and no simple, rapid techniques of direct sampling have yet been found that will establish population means with the precision required in intensive studies. So the study of a defoliating insect on a series of permanent plots requires a large field staff

and, over a period of years, represents a considerable investment. A large program of direct sampling can only be justified when the objects of the work cannot be satisfied by simpler methods based, perhaps, on relative population or population indices.

The objects of the Green River studies on the spruce budworm have been described, as well as the reasons for developing comprehensive life tables in a variety of forest stands (24). Life tables must be based on absolute population so that they are influenced only by real changes in insect numbers. Also, it is not sufficient to study mortality factors alone and to start each life table with a hypothetical population of 100 insects. From such life tables it is not possible to determine how the effectiveness of different mortality factors is influenced by changes in population level. Again, some mortality factors are so difficult to study that they can be assessed only by measuring changes in absolute population from one period to another. Secondary objects of the Green River work include the determination of population intensity, for use in relation to defoliation and damage studies, and the estimation of population per acre, for comparison to the populations of bird and mammal predators. Early in the course of the work it became clear that these objects could only be met by the use of an appropriate basic unit, supplemented by enough data on the structure of the tree and the forest to permit conversion of results to population intensity and to absolute population. The supplementary data can be gathered at any time of the year and need not occupy personnel during important sampling periods. It will be shown below (Sec. 13) that the basic unit selected—the whole branch—is in itself a reasonably good unit of absolute population measurement over a period of several years.

In brief then, considering the objects of the spruce budworm investigation, a program of direct, intensive sampling seemed amply justified.

4. The Timing of Sampling

4.1 General Considerations

The decision having been reached that an intensive sampling program can be justified, it is now important to decide how sampling should be timed, in relation to the life history of the insect, to provide the most useful results. In fact, the nature of the life history and the location of each stage (foliage, bark, wood, ground, etc.) will often be important factors in deciding whether or not sampling is feasible and if so, for what stages of the insect. Life tables for human populations are based on a constant age interval but insect life tables will generally be based on irregular intervals dictated by the exigencies of sampling.

There are several factors to consider in selecting the best sampling periods. For instance, a stage in which the insect is relatively large and easily found is easier to sample than a small, concealed stage, other factors being equal. Stages in which the insect is relatively stationary in feeding sites or resting places lend themselves to sampling but stages in which flight or dispersion is taking place do not. Also, larvae of defoliating insects may be firmly

attached to the foliage in some instars of development, through the nature of their feeding sites, but very prone to drop from the foliage on the slightest disturbance in other instars. Under the latter conditions great care is necessary in collecting sample branches or twigs without losing some of the insects. Another factor of importance is the stability of the population in time. During certain periods in the life history of an insect the population declines very rapidly from day to day. If the object of the sampling is to compare the population level on a series of plots, such periods should be avoided unless all plots can be sampled on the same day. Forest inventory data become obsolete in a few years through the action of tree growth, decay, and mortality; insect population data, if gathered at a dynamic point in the life cycle, may become obsolete in a matter of hours. Fortunately, insects have quiescent stages, and periods of relative population stability may occur even during the active larval stage. These periods may permit the collection of comparable population data over a period of days or even weeks. Also, the storage of foliage samples at near-freezing temperatures can be used to arrest development and mortality during active periods, until time is available to find and record the insects. A final consideration in the timing of sampling concerns the incidental data that are sometimes obtainable on natural control factors. For example, the sampling of pupal cases after emergence of the adult may reveal the proportions of pupae killed by parasites, invertebrate predators, and disease, whereas the sampling of living pupae would not provide this information.

The selection of appropriate sampling periods depends upon the insect to be studied, its life history and habits, the site where each stage is found, and the suitability of the site for sampling purposes. In any one year, the calendar timing of sampling is dictated by the rate of development, which may be determined through general collecting of the insect itself or through reliable phenological indices. If the general study area is large enough to include plots that are phenologically different, the timing of collections should be adjusted accordingly.

4.2 The Spruce Budworm

The life history of the spruce budworm was discussed in the first paper of the series and a graph was presented to show a typical population trend from egg to adult (24, Fig. 1). This information, when considered in relation to the factors outlined above, suggested that the following stages might lend themselves to intensive population sampling.

Eggs

The egg stage represents a period of relative population stability. The egg masses are rather firmly cemented to the needles and are not easily dislodged during the collection of the foliage samples. Also, as the empty masses remain on the needles after hatching, population sampling provides useful incidental data on sterility and on egg mortality caused by parasites and small predators (24). The attendant disadvantage of egg sampling is the relatively small size

of the masses, and the fact that they may be deposited on foliage of any age and on either the upper or lower surfaces of the needles. The difficulty of finding all the masses during examination of foliage samples necessitates constant checking to minimize the human error (Sec. 5.3).

Second-instar Larvae in Hibernacula

Overwintering larvae in hibernacula present a long period of relative population stability, and an important period to sample for the purposes of obtaining complete life tables. Unfortunately, the hibernacula are very small and concealed in staminate flower bracts, or under bud scales and lichens. It is too time-consuming and inaccurate to assess population by direct searching, but the larvae can be induced to emerge from the hibernacula and collect at a source of light in special laboratory cages. Techniques for this type of sampling will be detailed in a later paper.

Second-instar Larvae in Mines

Upon emergence from the hibernacula in May, second-instar larvae make feeding mines in staminate flower clusters, vegetative buds, or old needles. The flower clusters, which are produced in abundance by balsam fir about every second year until severe budworm feeding has damaged the shoots too much (21), are normally preferred. The presence of webbing and frass, and the rapid discoloration of mined needles, provide good visual clues to the location of mines. However, sampling during this period is inclined to be time-consuming, particularly in a year of heavy flower production, and is best suited to a few very intensive plots where complete trends of larval population are required.

Third-instar Larvae in Opening Buds

In the third instar the larvae generally migrate to the opening vegetative buds. Once they have established feeding sites in the new shoots, there is a period of several days of relative stability in population during the third and early fourth instars (24, Fig. 1; 17, Fig. 2). The larvae are too small to be very attractive to birds and no parasites cause mortality in this period. The nature of the feeding sites is such that foliage samples may be collected, with ordinary precautions, without much risk of losing larvae. The larvae are easy to find and sampling is greatly facilitated by the necessity of examining only the new shoots, rather than all the foliage on the branch (except under conditions of heavy flower production, when some larvae may remain in flower clusters until the flowers have dried up). This is an ideal period for intensive population work on all types of plots, providing it is carefully timed and not extended into the later instars where populations decline rapidly.

Fourth- to Sixth-instar Larvae

In these instars, and particularly the later ones, the larvae are more active and tend to move back to older foliage as the new foliage is exhausted. In old, severe infestations, where the crowns of the trees have become very thin

through progressive defoliation, they may exhibit even greater movements. Under these conditions direct sunlight may overheat the larvae and cause a movement towards the trunk of the tree, or photic reversal brought about by progressive starvation may produce dropping to the lower parts of the tree or to the ground (35, 36). The latter phenomenon occurs when a discrete source of light (the disk of the sun) is visible; the larvae remain photopositive to the diffuse light of an overcast sky (35). During these three instars, and particularly the sixth instar, the larvae are also very prone to drop on silk threads when the branch is disturbed. A further factor affecting population comparisons arises from the fact that the population is declining very rapidly through the action of parasites, predators, disease, and other factors (17, Fig. 2). However, if special precautions are taken to prevent loss of larvae during collecting (Sec. 5.1), and if collecting in old infestations is confined to cool, cloudy weather, population work can be continued during this period. Sampling should be carefully timed to provide the most useful data on parasitism (17) and permit the plotting of a population trend between the third-instar larval stage and the pupal stage.

It is for such periods as this that the frass-drop method can be particularly valuable, for it permits the derivation of larval trends with little effort and with no disturbance to the larvae themselves. In the course of the Green River work a technique has been developed whereby frass measurement alone, without complementary larval collection, can be used to establish such trends. Although the retention of frass by budworm webbing limits its use for the spruce budworm, the technique will be the subject of a later paper in the present series.

Pupae

The pupal stage is quiescent and the ease with which the pupae may be found on the foliage samples encourages rapid and accurate sampling. As the cremasters of the pupae are generally attached to the foliage by silk strands, the empty pupal cases remain on the foliage after the moth has emerged. There are several advantages in delaying sampling until the majority of the cases are empty. Examination of the cases provides useful data on natural mortality factors for the life tables. Also, the population of empty cases is relatively stable until they become broken up and dislodged from the foliage by wind and rain (Sec. 4.4). A disadvantage of such delayed sampling, but not a very serious one, is the fact that the predation on living pupae by birds must be recorded as though it had occurred during the late larval instars (24).

Moths

Methods for measuring active moth populations on an absolute basis have not been devised. Methods of assessing relative abundance, involving the mark-and-recapture technique, the use of light-traps, and the caging of virgin females on tanglefoot boards, have been attended by only limited success but will be described in a subsequent paper.

The population work described in the present paper applies to the egg stage, the larval stage (particularly the third instar), and the pupal stage.

4.3 Retention of Egg Masses on the Foliage

In sampling hatched egg masses, or empty pupal skins, we are really sampling insect signs rather than insects. Although these signs have biological stability because they are not destroyed by parasites or predators, they may lack mechanical stability. Because of their fragile nature, they are gradually broken up and dislodged from the trees through the action of wind and rain. This gives rise to two sources of error in population studies: (1) a negative error resulting from the loss of new egg masses or pupal cases between the date of their formation and the date of sampling; (2) a positive error resulting from the inclusion of old masses or cases that have been retained on the foliage for a full year or more. These two sources of error will be discussed separately.

To determine the loss of new egg masses of the spruce budworm, population sampling was carried out as soon as oviposition was completed and was repeated, using the same trees, about ten days later when practically all the masses had hatched. Ten trees were used on each plot, with sample branches selected and examined by methods to be described below. Some plots showed slight decreases in population and others showed slight gains, none of the differences being statistically significant. The loss of new egg masses in a 10-day period is sufficiently small, therefore, to be obscured by sampling error when based on 10 trees per plot. More direct experiments were then conducted in which mated female moths were caged on the branches of trees. The cages were removed when oviposition was completed and the position of each mass was plotted on a schematic diagram of the branch so that each mass could be identified in subsequent examinations. This work was conducted over a period of two years and a total of 125 egg masses was plotted. The mean loss, based on the original number of masses, was 3.6% in one week, 5.7% in two weeks, 7.5% in three weeks, and 9.3% in four weeks. Although it is possible that some of the missing masses were removed by birds, the progression of the loss in the weeks after hatching suggests that it was principally mechanical. If sampling is carefully timed according to the majority of the masses, i.e., not delayed until all the late masses have hatched, errors arising from this source should not generally exceed 5%.

The second type of error, resulting from the retention of masses for a year or more, is somewhat more troublesome. The same numbered masses used in the experiment described above were left until the egg sampling period of the following year, when it was found that 20.8% of the original number were still retained on the foliage. Also, on a number of population plots, sampling for old masses was conducted immediately before any new masses were deposited. The percentage retention, based on the total (new plus old) masses found on the same plots in the preceding year, was as follows: 1952—22.0%, 1953—25.1%, 1954—25.8%. If the same figures are based on only the new masses found in the preceding year, they increase to about 35% in 1954. This suggests that some masses are retained for two full years or more.

Thus, as an infestation progresses, there is a gradual accumulation of old masses until the later stages are reached, when the process is probably reversed by the destruction of old masses through larval feeding on old foliage and through the dropping of needles from drying twigs and branches.

Fortunately, new and old egg masses can generally be separated by their appearance. Newly-hatched masses are translucent and shiny and stand erect on the needles (Fig. 1). Old masses, in general, have a gray, opaque appearance and are collapsed, lying more or less flat against the needle; often, they are partially eroded (Fig. 2). However, some of the old masses, occurring on particularly well-sheltered parts of the branch, retain the translucent, erect appearance for a full year. A careful examination of 526 old masses collected from various plots just before the oviposition of the current year showed that 14.0% of them were sufficiently erect and translucent to be confused with newly-hatched masses. The magnitude of the error that is thus introduced to population studies, where the main object is to find the number of new masses deposited each year, will depend upon a number of factors and cannot be evaluated exactly. If the number of eggs deposited in the current year is equal to the number deposited in the preceding year, and if there is 25% retention of the latter, of which 14% remain indistinguishable from current eggs, then the positive error in establishing current population is 14% of 25% = 3.5%. If we are dealing with a rising population the error will be lower than this, and if we are dealing with a declining population it will be higher. A population decline of 50% would increase this error to 7.0%, and a decline of 75% would increase it to 14.0%. Because of the other factors involved, these percentages are only approximations and should not be used as correction factors. For example, the disappearance of some of the new masses before sampling, as discussed above, and the collapse of some of the earliest masses to hatch so that they resemble old masses, are factors which tend to compensate for the positive error introduced by old masses. Also, all masses found on new needles must, of course, be new masses regardless of appearance.

The following precautions can be used in budworm population work to minimize such errors: (1) careful timing of sampling; (2) separation of new and old masses in all collections by one experienced worker; (3) sampling on certain plots both before and after the oviposition period to determine whether any apparent difference in the population of old masses, suggesting errors of segregation, has occurred; (4) in rapidly declining populations, advancement of the sampling date to the time when most masses are still unhatched (some masses hatch before all masses have been deposited), with a later collection from the same plot for use in assessing egg mortality. With these precautions, it is possible to keep errors low enough for most purposes.

4.4 Retention of Pupal Cases on the Foliage

The two types of error, one negative and one positive, apply to empty pupal cases as well as to hatched egg masses. The only difference is that we are not concerned with the loss of living pupae from the trees. Living pupae are attractive to insectivorous birds, which consume case and all, and a small

proportion also drops to the ground, of which the majority are eaten by small mammals. Marked pupae left on the ground have suffered as high as 96% predation in one night. Pupal mortality caused by birds and mammals is included with larval mortality in life tables, because pupal sampling is delayed until mortality and moth emergence are nearly over. Thus the total mortality recorded from third-instar larvae to sampled pupae includes the pupae removed by birds and mammals (24).

The rate of loss from the trees of empty pupal cases of the spruce budworm was studied over a period of five years by several methods. Larval and pupal population trends of the type shown by Miller (17, Fig. 2) become relatively flat for a week or so after moth emergence, indicating that the loss of pupal cases is small enough to be obscured by random sampling errors. Numbers of pupal cases occurring naturally on the trees have been marked and re-examined at weekly intervals. On some population plots each year, pupal cases have been recorded during egg sampling, which takes place about four weeks later than the pupal sampling on the same trees. As an additional check, fallen cases have been sampled on ground quadrats and compared to the population of retained pupae by conversion of both figures to population per acre. The results of all methods agree fairly well and indicate a weekly drop of pupal cases ranging between 4% and 9% and averaging 6.7%. The data reveal no significant differences between years, although the cases were exposed to heavy rains in some years. In view of this loss of cases, pupal sampling must be very carefully timed and should be delayed only a few days after the peak of moth emergence, even if a proportion of the late individuals are still in the pupal stage.

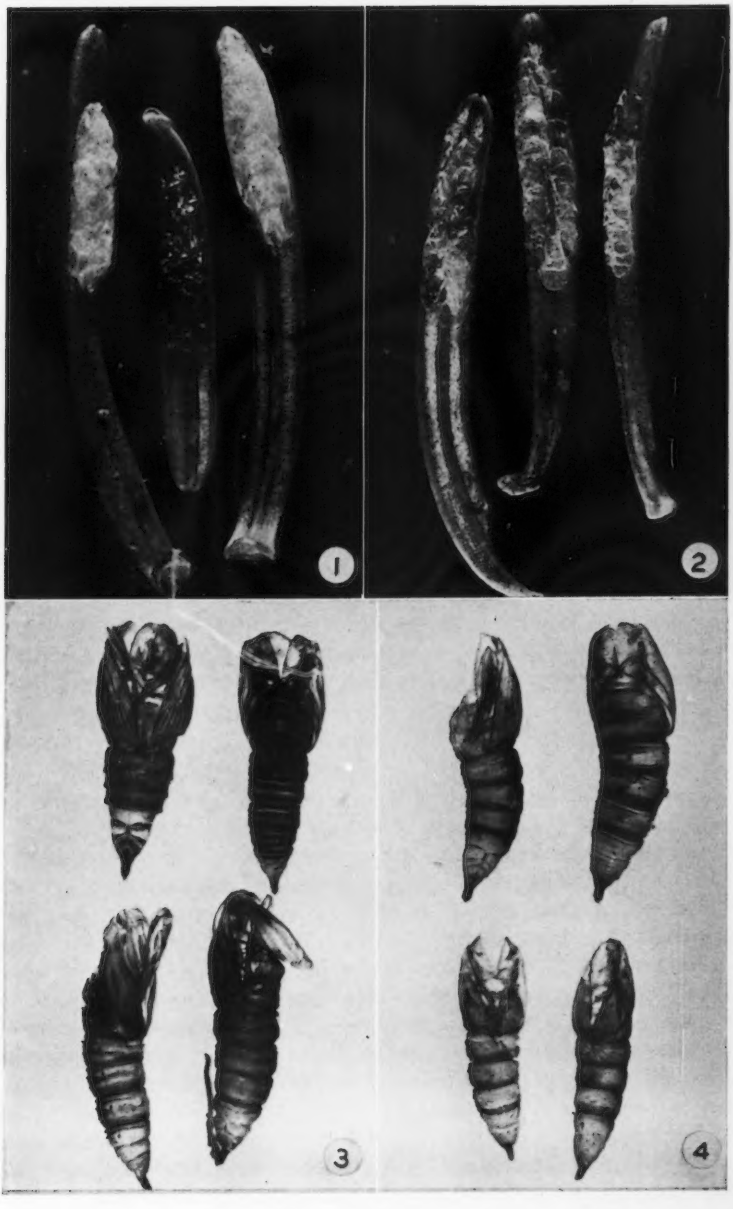
Of all cases (new and old) found during pupal sampling in one year, 15% are retained on the foliage to the pupal sampling period of the next year. This mean, which appears to vary little from year to year, is based on populations of old cases, measured just before new pupae are formed, as compared to populations on the same trees in the preceding year. If retention is based on only the new cases of the preceding year, this figure is increased under normal conditions to about 20%, suggesting that a few cases are retained for more than one year. As in the case of the egg masses, however, old cases can generally be recognized by their appearance (Figs. 3, 4). Of all cases retained for a full year, 85% become so bleached that they are readily distinguished from new ones. The location of the cases in reference to fresh larval feeding sites is a factor which helps to resolve many of the doubtful cases.

The same precautions recommended above for egg sampling can be used to keep the errors arising from pupal retention at a low level. Particularly useful is the recording of old pupal cases during larval sampling throughout

---

FIG. 1. Newly-hatched egg masses of the spruce budworm on balsam needles, and a black mass resulting from parasitism by *Trichogramma minutum* (Riley). × 7.0. FIG. 2. Old egg masses retained on the foliage for a full year. × 7.0. FIG. 3. Spruce budworm pupal cases from which moths have recently emerged. × 3.2. FIG. 4. Old pupal cases retained on the foliage for a full year; note bleached appearance and missing cephalic appendages. × 3.2.

PLATE I



the season. This not only makes sampling personnel familiar with the appearance of old cases, but serves to reveal any errors of segregation as the new pupae are added to the population.

Errors of both pupal and egg retention become particularly important when the budworm population has suffered a sudden and drastic reduction, such as that resulting from aerial spraying. Under normal conditions the number of old cases or masses that is indistinguishable from new ones is small in relation to the new ones; but when a sudden reduction is effected in the population, this number becomes large in relation to the new increment. To illustrate with an example from the field, a plot on the Green River Watershed had a pupal population in 1952 of 65.20 per sample unit. Aerial spraying of the larval stage in 1953, in addition to natural mortality factors, so reduced the population that only 1.61 new pupae per unit were found on the same trees. Of the 65.20 pupae in 1952, 15% were retained to 1953 and of this number, 15%, or 1.47 pupae, remained sufficiently dark in color to be confused with new pupae. Unless special precautions had been taken, the estimate of pupal population surviving the treatment could have been in error by nearly 100%.

5. The Mechanics of Sampling

The mechanics of collecting the sample branches and of finding and counting the insects must receive early attention, of course, in the development of any sampling technique. The procedures to be used for forest insects depend upon the nature of the stand, the size of the trees, and the life history of the insect. For some insects, it is necessary to draw samples from both the tree and the forest floor; for the spruce budworm it is necessary to draw samples only from the tree.

5.1 Collecting the Sample Branches

For reasons to be developed later (Sec. 8.3), it is necessary to collect sample branches from different levels of the tree crown, including the apical quarter. The most obvious way to do this is to cut down the tree so that the whole crown is readily accessible. The felling method was used on the Green River Project until 1949, when it was discarded in favor of superior methods. In addition to a number of rather obvious disadvantages, such as the loss of wood and the effect on plot density, felling has two important disadvantages of a more technical nature. A proportion of the insects is dislodged from the foliage when the tree strikes the ground, so it is necessary to fell the tree in a cleared space, where a large sheet of heavy canvas has been spread to receive the crown and permit counting of the insects dislodged. The percentage dislodgement is very variable from tree to tree, as indicated by Green River records between 1946 and 1949, and the use of a mean correction factor to obviate the use of the sheet would be attended by considerable risk of error. Dislodgement also varies with the insect stage, mass, feeding site, and method of attachment. For egg masses and early-instar larvae of the spruce budworm

it is generally below 5%; for late-instar larvae and pupae it may sometimes exceed 50%. The insects recovered from the sheet represent dislodgement from the whole crown. However, it is statistically unsound to count the insects on every branch in the tree (Sec. 12.2). The selection of sample branches from felled trees is subject, therefore, to two important restrictions: (1) for each tree, the sample branches must represent an exactly known proportion of the whole crown, and this can only be achieved by the laborious counting of all branches and the mechanical selection of each sample branch at a constant interval during the count (every tenth branch, for example). (2) As population on the sample branches must be corrected in proportion to the number of insects dislodged from the whole crown, the sample branches must represent a higher proportion of the crown than is statistically desirable for other purposes (e.g., if 1000 insects are counted on the canvas sheet, one would hesitate to say what proportion of them came from any one branch, or from a 1% sample of the crown; but one might be willing to assume that 20% of them came from a 20% sample of the branches). The other technical objection to felling is that a tree can be sampled only once after it is felled. For spruce budworm populations (and this is probably true of most forest insects) the major source of variance is intertree, and if successive samples can be drawn from the same standing trees they are often found to be significantly correlated (Sec. 10). There is thus a statistical advantage in sampling standing trees, for changes in population from one period to another can be measured with greater sensitivity. These restrictions imposed by the felling method were a deterrent to the development of sound sampling techniques.

Tree climbing with the aid of climbing irons was even less successful than felling. The dense nature of balsam crowns makes climbing difficult and the disturbance caused by climbing, and by cutting the branches and getting them to the ground, made the method quite unsuitable for active or for easily-dislodged stages of the budworm.

The problem was finally solved by the use of a 38-ft. aluminum extension ladder, along with an aluminum pole pruner. The ladder is equipped with a folding brace, which holds the climber about 8 ft. from the trunk of the tree and permits unobstructed access to any part of the crown (Fig. 5). It is steadied by three guy ropes which may be attached to nearby trees. The pole pruner is assembled by the operator from 5-ft. interlocking sections, according to the length he requires. The cutting head is a conventional pulley-and-leverage type, to which has been added a pair of clamping jaws that grip the cut branch until the operator is ready to let it drop to a canvas sheet under the ladder (Fig. 6). The most efficient ladder crew consists of five men: a climber who operates the pole pruner; a man who steadies the base of the ladder while it is raised, then raises the ladder extension to the desired height, and later stands back and directs the pole operator into the desired crown levels; and three men who hold the guy lines until the ladder is in position and then converge at the canvas sheet to measure the sample branches as they are dropped, clip them into small pieces for convenient

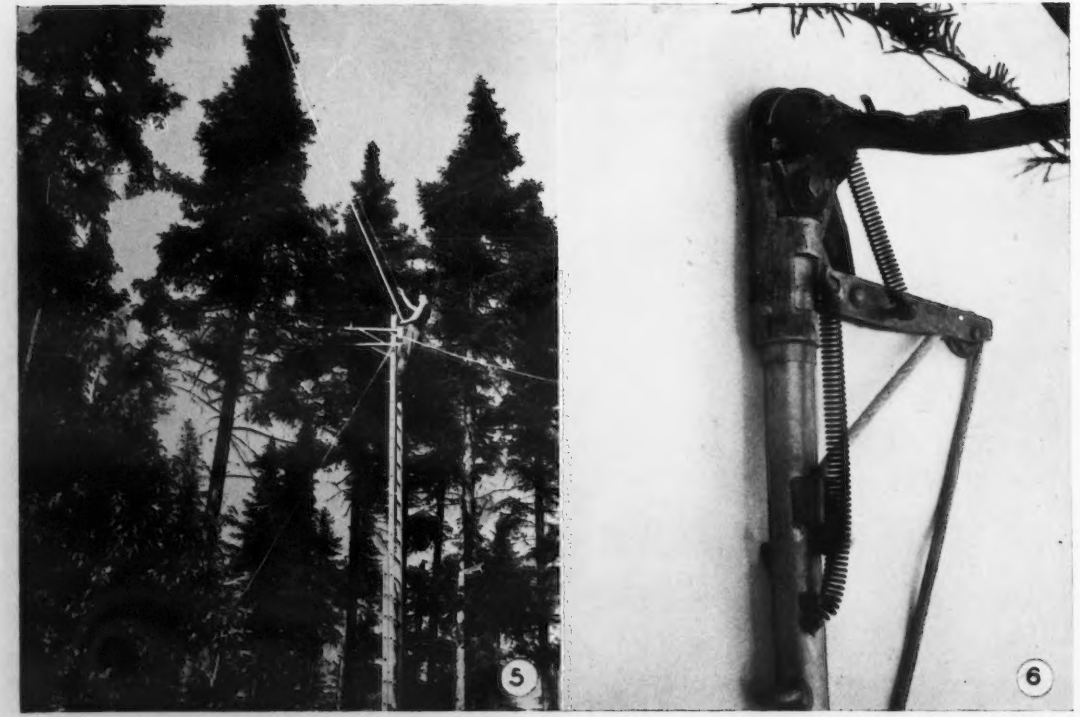


FIG. 5. Operation of aluminum extension ladder and pole pruner in collecting sample branches from tall trees.
FIG. 6. Cutting head of pole pruner with cut branch held by clamping jaws.

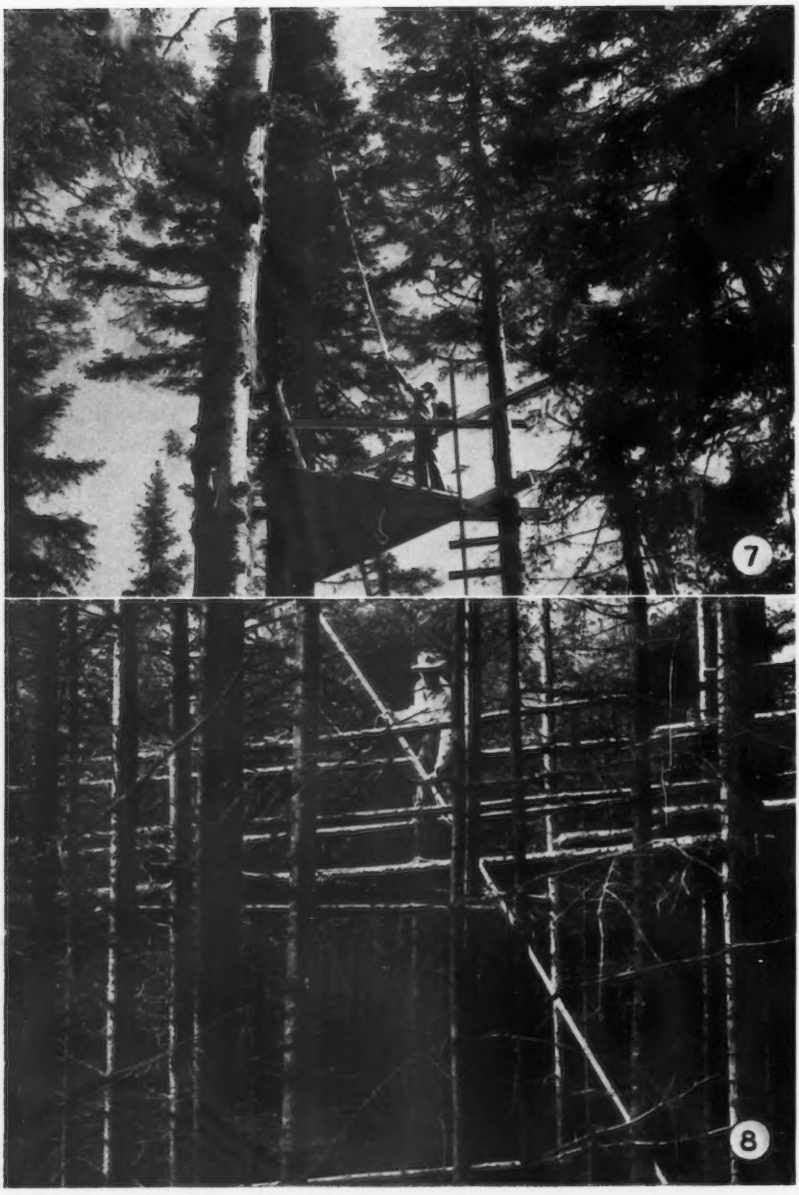


FIG. 7. Tree platform used in collecting frequent samples from a group of trees.
FIG. 8. Trestle used in collecting samples in dense stands.

sampling, and pack them in canvas bags. A crew of four men is often sufficient. The operation of the ladder has been described in more detail by the writer (20), with the statement that 15 to 20 large, mature trees could be sampled at four crown levels in a day by this method; in 1954, with an experienced ladder crew, as many as 25 trees were sampled per day.

With the ladder and pole pruner, samples are readily obtained from the desired levels of trees up to 70 ft. in height, which is greater than the usual height of mature balsam fir. For younger trees, up to 35 ft. in height, the pole pruner can be used from the ground. Certain population plots in mature stands may be far enough from a motor road that time is lost in carrying in the ladder on sampling dates. Also, on plots that are to be sampled at intervals of a few days to determine larval population trends, it is not always convenient to use a full ladder crew. These special situations can be met by the construction of tree platforms (Fig. 7), which may permit the sampling of five or six trees each if carefully located, or of tree trestles (Fig. 8), which may be extended any desired distance through the plot. Trestles may be constructed from pole-size material cut on the spot (Fig. 8), or from steel cables. They require fairly dense stands so that attachment to adjacent pairs of trees is possible. Trees suitable for platforms can be found even in old, rather open-growing stands. Construction work of this kind should be done in the early spring, before development reaches a stage where the jarring of the trees causes any dislodgement of insects.

5.2 Examining the Foliage

Measurement of the insect population on foliage samples is commonly done by ocular examination, the worker endeavoring to find, remove, and record each insect. In the early years of the Green River studies this was done on the population plot, where the worker was subject to such discomforts as biting flies and inclement weather. Experience soon showed that greater accuracy could be obtained by packing the foliage samples in canvas duffle-bags, which could be tied tightly at the top to prevent any insects from escaping, and transporting them to the laboratory for examination. At dynamic periods in the life history of the insect, when the risk of population changes makes it necessary to sample all plots in a period of a few days, the bags can be stored at near-freezing temperatures until there is an opportunity to examine the foliage. Even under the best of conditions, the examining of the foliage is extremely tedious, particularly when eggs or early-instar larvae are being sampled, and is subject to personal error. One type of error, which may be either positive or negative, results from miscounting the insects, or even forgetting the count before it is recorded. This type can readily be obviated by providing each worker with a small desk counter or hand counter. The other type of error, and by far the more common type, is the missing of some of the insects through lack of thoroughness and patience in examining the foliage. Check examinations are usually desirable to permit the assessment of such errors. Many factors can contribute to personal errors of this type, and constant vigilance is required to keep them at acceptably low levels.

Important considerations in reducing such errors are adequate supervision, the maintenance of high morale among the workers, recreational breaks to relieve tedium, comfortable working facilities with adequate lighting, and the fractioning of branch samples into small twig segments not more than 2 or 3 in. long. Insects are much less likely to be missed on a small twig that can be rotated in the fingers than they are when the worker attempts to examine a whole branch as one unit.

The examination of foliage samples, in addition to being subject to personal errors, is also the most time-consuming part of sampling Examination is therefore the limiting factor in determining how many plots, and how many trees per plot, can be sampled in a season with the available staff assigned to the project. I believe that the greatest need in the further development of sampling techniques for defoliating insects is for some purely mechanical, chemical, or electrical process for measuring insect abundance on a given quantity of foliage. Specialized apparatus for mechanical sorting has greatly stimulated work on the populations and ecology of soil insects. A corresponding development for foliage insects, which would measure animal protein or some factor closely associated with insect numbers, would permit real advances in forest insect ecology.

5.3 Checking the Foliage

In general the amount of checking required depends on the organization of the work, the quality of the workers, the degree of supervision, and the relative concealment and size of each stage of the insect. In the larval and pupal sampling at Green River, a formal check examination of all foliage has been considered unnecessary. There are six senior men who are responsible for various aspects of the budworm investigations, and most of these aspects are closely integrated with the population work and the development of life tables. During sampling periods all these men assist with both the actual sampling and the supervision, and all have a real interest in the maintenance of a high degree of accuracy in the population data. In the larval and pupal sampling, two or three of the more casual workers are assigned to work with each senior man, who makes frequent visual checks on the thoroughness with which the examinations are made. With this type of organization and supervision, it is believed that a complete check examination of all samples may be dispensed with for relatively large stages such as larvae and pupae. In situations where the sampling establishment consists almost entirely of casual workers, with limited supervision, complete check examination on at least a considerable proportion of the samples would be highly desirable.

For spruce budworm egg masses, which are small and easily missed, formal check examinations are necessary regardless of the quality of sampling personnel. It was learned early in the course of the present work that only limited confidence could be placed in egg population data when thorough check examinations were not made, and it has been a consistent policy to have each foliage sample examined by one worker and then re-examined by

another. The senior men, who have a real concern with the accuracy of results, are generally selected to make the check examinations. When it is necessary to conserve time, a 50% check is often made on each bag of foliage and the result doubled. In this case, the checker examines only every second twig as he draws twigs from the bag.

Information on the percentage of eggs likely to be missed in the first examination has already been presented by the writer (22), and it will be necessary only to summarize the main conclusions here, as based on records for various plots over a period of five years:

1. The percentage miss is related to population level and may vary from 40% in very light infestations to about 12% in severe infestations. This is a rather surprising phenomenon that has been demonstrated consistently from year to year.

2. The percentage miss is also related to the nature of the foliage. It tends to be lower in young stands with vigorous shoot growth than in old stands where the needles and shoots are more compact.

3. Proficiency in egg sampling varies considerably from one worker to another. It is lowest in inexperienced workers, before they realize from the results of checking how easily eggs may be missed, and in such cases may exceed 50%. Except in rare instances, experienced workers seldom succeed in reducing their errors below 10%, even under conditions of high population.

4. For any one examiner, the percentage miss is not constant from plot to plot or from year to year.

Considering these results, there would appear to be little hope that a simple correction factor could be obtained and applied to egg population data to eliminate checking. Correction factors would have to be specific for the worker, the plot, the population level, and the year. Also, removal of the foreknowledge of checking would undoubtedly affect the errors of many workers. Prebble (29) reached the same conclusions with regard to check sampling for cocoon populations of the European spruce sawfly.

As egg sampling is the most time-consuming aspect of the population work, any short-cut or device that may possibly expedite it is worthy of investigation. It was considered in 1950 that possible preferences of the female moths in selecting oviposition sites might provide a clue to such a short-cut, so the location of several hundred egg masses was recorded as to age of foliage, upper or lower surface of needle, length of needle and portion (apical or basal) to which the mass was attached. The distribution of foliage quantities, such as total shoot numbers and total weight of needles, was also studied and chi-square tests were applied to determine whether the distribution of eggs, according to age of foliage, was significantly different from the distribution of foliage quantity. In the interests of brevity, only the conclusions will be summarized:

1. The distribution of egg masses is significantly different from the distribution of foliage quantity, whether foliage is expressed as shoot numbers or as total needle weight. There is a tendency for eggs to be deposited on needles

of the more recent years, and over 90% are deposited on needles that are less than five years old. However, because of the influence of flowering on foliage growth (21), there is no consistent pattern. If the current year is a non-flowering year for balsam, the new foliage may represent 20% of the total foliage and may bear as high as 55% of the egg masses; if the current year is a flowering year, with reduced shoot production and short, closely spaced needles, the new foliage may bear only 15% of the masses. Distribution of masses becomes even more irregular as severe defoliation of new foliage is experienced, so that the proportion of eggs on older foliage must increase.

2. The chi-square test indicates that the proportionate occurrence of egg masses on upper and lower needle surfaces does not differ significantly from 50 : 50.

3. Most egg masses are found on needles of average length and the short needles associated with heavy flower production are generally, but not always, avoided as oviposition sites. Practically all egg masses (over 99%) are found on the apical half of the needle.

In view of these results, it is clear that no system of partial sampling, such as the examination of current foliage only and application of a conversion factor to obtain total egg masses, is feasible. All foliage on the branch, and both needle surfaces, must be examined.

6. Selection of the Universe

Most statisticians use the words 'universe' and 'population' synonymously to refer to the entire number of individuals from which a sample is to be drawn. In forest insect sampling, the universe is actually the sum total of the insects of the given species in a specified area of forest land, or in a defined habitat within the specified area. However, as the universe must be defined in terms of trees, foliage, or forest floor, it has become common practice to refer to the stand or the habitat as though it were the universe. In other words, the term 'universe' is used to represent the habitat in which the 'population' occurs. Similarly, the sample unit and the sample are defined in terms of foliage or forest floor, since the selection of samples must be based on these habitat units. This broad use of terms does no particular harm, and serves a purpose in the avoidance of much awkward phraseology when the universe and the sample are described. (In one sense, the insect population represents a *property* of the trees, as does tree diameter or age, and in this sense it is quite proper to call the trees the universe.)

Whenever we are confronted with a sampling problem, it is of early importance that the universe be carefully defined in order to eliminate the danger of applying conclusions to a broader or more heterogeneous universe.

The Green River investigation on the spruce budworm is concerned with a watershed containing about 400 sq. miles of forest land. The determination of the population on the whole watershed by random sampling would obviously be impractical and, in any case, is not required within the defined objects of the study. We may next consider the major forest types and age classes within

the watershed, each of which has irregular boundaries and sizes depending upon the action of major forest influences in the past. Typical examples of these conditions are mature balsam stands originating after wind storms between 1850 and 1875, young balsam stands originating after the spruce budworm outbreak that started in 1912, mixed stands of balsam and spruce originating after a forest fire in 1899, and mixed forests of hardwood and softwood. Within one of these major types there may also be different forest conditions arising through recent silvicultural experiments or cutting practices. Again, fortunately, the objects of the study do not require an estimate of total budworm population for the whole area of a given forest type, but only that population dynamics and natural mortality should be studied under different forest conditions that can be adequately described and compared. Any stand, characterized by homogeneous forest conditions throughout its area, may therefore be considered a sampling universe, with the number of stands selected for study depending on the available staff. *On an area basis*, therefore, the problem is one of sampling from a number of homogeneous conditions rather than a problem of stratified random sampling from a heterogeneous universe. Although, in one sense, the different forest stands to be studied represent strata of the whole forest, it would be confusing to define them as such because no stratified sampling design for stands is intended. The objects of the study do not require a random distribution of samples within a broad forest type, or a sampling intensity that is proportional to the occurrence of each type. In any case, the elegant sampling designs that are used effectively in agricultural crop experiments would be quite impractical at the present stage of development of forest insect population techniques. We must depend upon rapid survey methods and such indices as defoliation and frass-drop to confirm, in the extensive sense, the conclusions derived from intensive plot studies.

As the stands for intensive population sampling are not selected by random methods, it is necessary to outline the principal factors that determine their selection. The criterion of homogeneity has already been mentioned. To ensure that all work is done in a homogeneous forest condition, a study area of about 25 acres is carefully located within the stand and delineated by painted lines. Study areas or plots of this size are small enough to be contained within homogeneous conditions and large enough to permit census work on insectivorous birds and small mammals. Plots are located well within a uniform forest condition, rather than on the edge, except in the case of plots especially established to study population dynamics in isolated stands. In general, each plot is typical of one of the important forest conditions on the watershed, although it does not represent a random sample of the condition. The plots are also selected to represent widely different conditions so that eventual comparisons of population dynamics in relation to such factors as stand age and density can be based on broad ranges of differences. The pattern of the infestation plays a part in plot selection, some plots being selected to represent the epicenter or focal point and others to represent stands infested more recently. Practical considerations in plot location include accessibility, and

the progress of cutting and spraying operations. There is no particular merit in walking several miles to sample a plot when the same forest condition is available near a motor road; and a plot in an area scheduled for harvest operations or control operations can serve only one specialized function.

Each life table, then, is developed for the universe represented by one homogeneous forest stand, but in devising sampling methods it is necessary to describe this universe more exactly. As all the budworm stages selected for sampling are found on the foliage, the universe for sampling could conceivably consist of all the foliage in the stand, regardless of the trees on which it occurs. If population did not differ significantly from tree to tree, this approach would be logical. Careful studies of variance between trees and within trees are therefore an early necessity in the development of sampling techniques for forest insects. For the spruce budworm, the intertree variance in population is nearly always much higher than the variance that occurs from side to side of the same tree, even when sampling is restricted to trees of the same size and crown class (Sec. 8.1). Population per sample unit shows even greater differences between trees of different crown classes (Sec. 9.1). So if we ignore the tree, which is the major source of variance, we are confronted with the problem of securing representative foliage samples from different types of trees in such a way that the amount of foliage in the samples will be proportional to the occurrence of the different trees in the stand. Variability from sample to sample would be very high, and we would also lose the statistical advantage that arises from the correlation between successive samples from the same trees (Sec. 10). There is a considerable advantage, therefore, in thinking of the universe as a universe of trees rather than a universe of foliage. Within this universe of trees, stratified sampling can be used to advantage when the objects of the study require an expression of absolute population. For the spruce budworm, stratification according to crown class is the logical choice; for other types of insects, stratification according to some other criterion might prove more appropriate.

Sampling for the purpose of estimating relative population only, or sampling for purposes of forest insect survey, should generally be confined to one stratum of trees. In fact, any sampling problem that does not require the measurement of absolute population can generally be solved to best advantage by using only one stratum, or, in other words, by severely restricting the universe as illustrated by Prebble (29) and later by Morris (19).

The fact that the variability in insect population between trees is so much greater than the variability within trees (i.e., between different sides or quadrants of the same tree) may well prove to be a principle common to all forest insects. It is true of all species that have been studied in sufficient detail in Canada, viz., spruce budworm (Sec. 8.1), European spruce sawfly (29), larch sawfly (16), winter moth (26), large aspen tortrix (30), lodgepole needle miner (34). The foregoing are all defoliating insects, but the habits of most boring insects would lead one to suspect an even stronger relationship. This relationship will be dealt with in more detail for the spruce budworm

(Sec. 10). Meanwhile the corollary that sampling designs may also have much in common, and may benefit through successive improvements as different insects come under intensive study, is worth keeping in mind.

7. Selection of the Sample Unit

We have established that the tree must receive recognition as the major source of variance in the universe. However, to examine all the foliage on the tree as the sample unit would be neither practical nor statistically desirable. In selecting a sample unit, therefore, we must give consideration to smaller units of foliage within the tree. Where the object is to assess absolute populations, selection of the most appropriate sample unit should be based on the following criteria:

1. In order for the sample to be representative of the universe, the sample unit should be of such a nature that all units in the universe have an equal chance of selection. This, of course, is a fundamental principle in sampling and is discussed further in Sections 8 and 9.

2. The sample unit must have stability. That is, the number of units available to the insect population must not be affected by changes in the growth habits of the plant caused either by intrinsic factors or by repeated insect damage.

3. The *proportion* of the insect population using the sample unit as a habitat must remain constant.

4. The sample unit should be reasonably small so that enough units can be examined on a given plot and date to provide an adequate estimate of variance. The other advantage of a small unit is flexibility, so that, after a certain amount of pilot sampling, the investigator will be able to choose the combination of units per tree and trees per plot that will assure the most economic allocation of sampling resources (Sec. 12.2).

5. In absolute population work, where estimates of population per acre are required, the sampling unit must lend itself to quantitative assessments of the total number of units per tree and per acre (Sec. 13).

6. An important practical consideration is the facility with which the sample unit can be delineated in the field and collected without a serious loss or disturbance of the insect population.

As new shoots constitute the major food supply of the spruce budworm, at least as long as they are available, it is logical that they should receive first consideration as a sample unit. Such a unit would satisfy most of the criteria, particularly those concerned with flexible sample size and ease of collection. Unfortunately, however, criterion No. 2 would not be satisfied at all. Shoot numbers can vary considerably with intrinsic factors such as flower production (21), and also from the effects of repeated budworm attack. This unit of population intensity must therefore be rejected as a unit for absolute population work, although it has been found useful for other purposes (9). More will be said of it later (Sec. 14.1).

The next choice might well be a restricted part of the branch. In Canada the use of the apical 18 in. of the branch in spruce budworm population work apparently originated in Ontario (2) for insect survey purposes, and has been applied in studies on flowering and non-flowering trees (4) and on the effects of aerial spraying (6). In the United States the apical 15 in. of the branch has been used in population work (7). These apical branch portions are sufficiently small to provide flexibility of sampling design and are easily collected. The main limitation of such units are that they fail to satisfy criteria Nos. 3 and 5. High temperature reversals can produce movements of the larval population towards the base of the branch with subsequent return to the exposed periphery when this is again in shadow (36). Depending on the time of collecting the samples, therefore, the apical portion of the branch may support different proportions of the larval population. Also, even casual observations during the course of a budworm epidemic will reveal that the apical portions of the branches become completely defoliated first, so that a gradual but distinct shift in the center of gravity of the population (eggs, larvae, and pupae) towards the interior of the crown must occur. Criterion No. 5 is not satisfied because, although the number of apical branch tips per tree and per acre can be determined, it would be very difficult to account for the basal components of the branches and for their insect population relevant to the tips.

These weaknesses point, inevitably, to the selection of the whole length of the branch in any serious attempt to study the population dynamics of the spruce budworm on an absolute scale. For the egg, larval, and pupal sampling to be described in the present paper, the bare branch stem need not be collected; for sampling overwintering populations, the bare stem and, in some cases, a sample of trunk bark should be included. The whole branch satisfies all the criteria, in general, but is somewhat more difficult to collect than either a given number of shoots or an apical portion. Large branches may have to be clipped in two or three sections, but with the pole pruner described above this presents no particular problem and can be done without serious disturbance to adjacent branches. Also, the whole branch represents a somewhat larger unit than is always desirable (Sec. 8.3). This has been solved by splitting branches *longitudinally*, i.e., by clipping all secondary branches from one complete side of the main branch stem and discarding the rest of the collected branch. The side to be retained is selected by random methods.

Branches vary considerably in size from tree to tree and from top to bottom of any one tree. To compensate for this, all branches are measured, to the nearest 0.1 ft., as to the total length of the foliated part and the width at mid-length. Only the side of split branches to be retained is measured for width. The product of these two measurements, in square feet, is termed branch surface. It is not, of course, an accurate measure of foliage surface, but merely a correction factor that serves a very useful purpose in permitting the direct comparison of insect populations on branches of different sizes. I wish to emphasize this last point, because it is a feature of the present

sampling technique that is easily misunderstood. The whole branch, or a longitudinal one-half, is the basic unit and the area measurement is only a correction factor; for tree species with a branch form different from that of balsam or spruce, the branch length alone or some similar measurement might provide a more useful correction factor.

The measurement of branch surface area would appear to be simple and objective, but it has been found that different workers may reach different interpretations as to the outline of the branch as well as the best way of handling branches that are forked, irregular, or barren of foliage in some parts. In order to minimize differences of this type it is important that all measuring should be done by the same man in the collecting party and that a set of conventions should be drawn up to cover all types of branches that may be encountered. These conventions should leave as little room as possible for the exercise of personal judgment. Branches that are partially or wholly bare as a result of budworm defoliation are measured, of course, as though they were fully foliated (criterion No. 2). As such bareness proceeds from the top of the tree downwards, and from the apex of the branches inwards, it is unlikely to be confused with the bareness resulting from the normal suppression of basal or interior foliage.

One of the greatest advantages afforded by branch surface measurement is the facility with which total branch surface per tree and per acre can be determined (Sec. 13).

8. Representativeness in the Tree

It has been concluded that the tree must receive consideration as a major source of variance in the universe, and that no sampling unit smaller than the longitudinal half of a branch is likely to provide a reliable basis for absolute population work. The next problem to be solved concerns the proper selection of branches and trees to ensure population results representative of the universe. Satisfaction of the criterion of representativeness is one of the most important steps in the development of sampling techniques for forest insects, and one that often receives too little attention.

The present section is concerned with the method of selecting branches, or half branches, in such a way that they will provide an unbiased estimate of population for a given tree. Before such a method can be established it is necessary to consider both the distribution of the insect population in the crown and the distribution of branches in the crown.

8.1 Distribution of Budworm Population

The distribution of the budworm population within the crown was determined in the course of inter- and intra-tree variance studies. Co-dominant balsam trees were selected randomly in groups of four, each group representing a different forest condition or a different population level of the budworm. The living crown of each tree was divided into four equal levels, designated *A*, *B*, *C*, and *D*, from the apical quarter downwards. The sample branches

were drawn from the middle of each quarter. The crown was also divided into four quadrants, in a directional sense, and these were designated *N*, *E*, *S*, and *W*, according to the four cardinal points of the compass. This provided 16 sampling loci within each tree, designated *AN*, *AE*, etc. One branch was collected, measured, and examined from each locus, except in the *A* and *B* levels where the smaller size of the branches in relation to their frequency made it advisable to use two. The two branches from a given locus in *A* or *B* were not treated as replicates; they were pooled. Quite by accident, this method of selection provided almost equal sampling intensity in the different levels, as will be shown below.

Eight groups of trees were sampled in this way and the results for each group were subjected to an analysis of variance (Table I). The field data were first tabulated as number of insects per 10 sq. ft. of branch surface (10 instead of 1, or 100, merely to provide for a reasonable number of digits before the decimal point) and were then converted to logarithms before analysis. This transformation, of the form $y = \log_{10}(x + 1)$, stabilizes the variance and makes the frequency distributions more nearly normal (Sec. 11), and this is necessary to satisfy one of the main assumptions underlying the analysis of variance (8). Each source of variance was tested against the second-order interaction (quadrants × levels × trees). In no instance, however, was any significant variance associated with quadrants or quadrantal interactions. Accordingly, quadrants were accepted merely as replicates and the sums of squares were pooled to obtain a stronger error term as shown in Table I.

TABLE I

EXAMPLE OF ANALYSIS OF VARIANCE IN THE STUDY OF INTER- AND INTRA-TREE VARIABILITY IN SPRUCE BUDWORM POPULATION

Source of variance	Sum of squares	Degrees of freedom	Mean square	*F*
Trees	1.3864	3	0.4621	5.25**
Levels	1.1099	3	0.3700	4.20*
Trees × Levels	0.4387	9	0.0487	
Quadrants	0.0672	3	0.0224	
Quadrants × Trees	0.7618	9	0.0846	
Quadrants × Levels	0.5449	9	0.0605	
Quadrants × Levels × Trees	2.8496	27	0.1055	
(All except trees, levels, and their interaction)	4.2235	48	0.0880	

* *Significant within the 0.05 level.*
** *Significant within the 0.01 level.*

Groups 1, 2, and 3 (Table I is based on Group 1) apply to larval populations on three different plots and dates. Population levels were between 25 and 50 larvae per 10 sq. ft., which is sufficiently low that no shortage of new foliage could affect the distribution of the population when the sampling was done. These groups show 'between trees' and 'between levels within trees'

as the significant sources of variance; in Group 2 the 'trees × levels' interaction is also significant. The variance associated with levels within trees, which in some groups is much higher than the intertree variance, is our main concern in the present section. In Group 1 the variance results from a concentration of larvae in the lower levels (19.1 per 10 sq. ft. in *A* to 31.9 in *D*); in Groups 2 and 3 it results from the opposite type of distribution (94.0 in *A* to 2.9 in *D*, and 91.5 in *A* to 17.7 in *D*, respectively). These differences in the vertical distribution pattern are difficult to explain. It has already been noted that scarcity of food at any level was not involved. It is natural to suspect that the top levels, which receive more light, may have more new shoots per unit of branch surface than the lower levels and that this may account for the Group 2 and 3 distributions. As a matter of fact, new shoots were counted on all samples and the analysis of variance repeated using number of larvae per 1000 shoots. The variance associated with levels was still highly significant but, as might be expected, was increased for Group 1 and decreased for Groups 2 and 3. Numbers of larvae per 1000 shoots were as follows: Group 1, 34.6 in *A* increasing to 71.4 in *D*; Group 2, 120.0 in *A* decreasing to 9.5 in *D*; Group 3, 100.7 in *A* decreasing to 52.5 in *D*.

Groups 4, 5, and 6 were used to study variance in egg population in three different stands. In all three stands, mean population was less than three masses per 10 sq. ft. These groups show a tendency for higher population in the *A* and *B* levels, but significance cannot be demonstrated. Also, significant intertree variance is demonstrated only for Group 4. At low population levels of the spruce budworm, errors of random sampling become very high in proportion to the population mean (Sec. 12.5). There is reason to suspect, not that distributional patterns are lacking at low populations, but that the high discrepance relative to the mean prohibits significant results when only a few trees are sampled.

Groups 7 and 8 were used to determine egg distribution under conditions of higher population. Group 7 (22 masses per 10 sq. ft.) had significant intertree variance and highly significant level variance, the latter resulting largely from a concentration of masses in the *B* level. Group 8 (140 masses per 10 sq. ft.) showed highly significant intertree variance and highly significant level variance, the latter resulting from a concentration of masses in *C* and *D*. Group 8 was the only group in which the distribution was influenced by budworm defoliation, the branches in *A* being almost completely defoliated and those in *B* being noticeably thin. As might be expected, there was interaction between trees and levels, probably resulting from the variable degrees of defoliation.

These results, together with more general observations during subsequent population work, warrant the following conclusions:

1. There are no significant differences in population associated with different sides of the same tree. Sample branches may therefore be collected from any one side, or from more than one side when replication in a homogeneous universe is desired.

2. There are substantial and significant differences from one crown level to another. In general there is a tendency for both larval and egg populations to be higher in the top levels (whether based on branch surface or shoot numbers) until defoliation in the upper levels causes the reverse to be true. In flowering years a concentration of small larvae has often been observed in the region where most of the staminate flowers occur (largely the *B* level). However, the vertical distribution does not follow a predictable pattern and one instance has been cited in which third-instar larvae were concentrated in the lower levels for no apparent reason. Also, the significant interaction between trees and levels in some groups indicates that the distribution by levels is not necessarily the same for all trees in a given group.

3. There are significant differences from tree to tree, even among trees of the same size and crown class. Under conditions of low population these differences may disappear, but it is more probable that they are obscured by random sampling error when only a few trees are studied.

8.2 Distribution of Branch Surface

Because of the important population differences among the four crown levels, it is necessary to determine how branch surface is distributed among the same levels. Otherwise it would be impossible to obtain a representative population mean for the tree except by selecting, for example, every tenth branch from the whole crown. Although such a selection method was used on felled trees in the early part of the study, it would certainly not be feasible on standing trees with the dense crown structure of fir or spruce. In any case, the estimation of population per acre requires data on branch surface per tree and it adds little to the work of crown analysis to keep separate records on the four vertical quarters.

This problem first arose in 1950 with the introduction of the sampling ladder, and data were then available on the crown structure of 55 trees which had been analyzed for other purposes (21). They were co-dominant trees from both young and mature stands and had been analyzed by 5-ft. intervals of crown length. By plotting branch surface over crown intervals for each size class it was possible to interpolate the appropriate foliage quantities for equal crown quarters. This indirect approach did not permit a statistical evaluation of the results. An additional 82 trees were therefore felled and analyzed by dividing the crown directly into vertical quarters (Table II). The second group contained approximately equal numbers of trees from several age classes of balsam fir, viz., 35 years, 55 years, 80 years, and 105 years. (All ages quoted in this paper represent age since initial release of the stand, for reasons already presented (18).) No significant difference in the percentage distribution of branch surface was detected between age classes. Trees in the 35-year class with crown lengths of 15 ft. or less had a tendency to bear a slightly greater percentage of their foliage in the apical quarter, but this was not sufficient to warrant the establishment of a separate distribution class.

TABLE II

PERCENTAGE DISTRIBUTION OF BRANCH SURFACE IN THE FOUR VERTICAL CROWN QUARTERS OF BALSAM FIR ON THE GREEN RIVER WATERSHED

Source	*A* Apical ¼	*B* 2nd ¼	*C* 3rd ¼	*D* Basal ¼
55 trees analyzed in 5-ft. vertical sections	7	22	34	37
82 trees analyzed in four vertical quarters	8.2 ± 0.3	21.3 ± 0.7	32.5 ± 0.6	38.0 ± 1.0

8.3 Representative Sampling

A decision as to one of three possible courses of action affecting the development of the sampling technique must now be made:

1. Each vertical crown quarter may be sampled separately, giving four estimates of population per 10 sq. ft., and these estimates weighted according to the distribution of branch surface to give one representative figure for the tree. This will provide data on the distribution of the insect in the crown through the course of the infestation. However, the field work, the examination, the record keeping, and the analysis of data will all be complicated and slowed by this attention to the four levels.

2. The crown level where population has a mean value, i.e., the 'center of gravity' of the population, may be determined and all sample branches drawn from this level. As the center of gravity may change from year to year, or even from day to day, the determination of its level on a given plot and sampling date will pose a serious problem. However, in rapid sampling work for extensive surveys, where only one level can be sampled, it is generally necessary to assume that the mid-crown level is representative of the tree (23). When this is necessary, the variance studies reported above suggest that the best results will be obtained by using the middle of the *foliated* crown, and thus lowering the sampling level as the upper levels become completely defoliated by the insect.

3. Sample branches may be so selected that the *intensity* of sampling is equal in each quarter. In other words, the amount of foliage collected from each quarter will represent the same proportion of the whole sample as the foliage of that quarter represents of the whole tree. The branches from all quarters of the tree may be put in the same bag and treated as a single unit throughout the remaining stages of the work. The estimate of population density for the tree is the total number of insects found in the bag of foliage divided by the total branch surface of all branches collected. This provides a representative estimate regardless of how the insect is distributed in the crown; the weighting process is simply taken care of in collection rather than in analysis.

It is not an object of the Green River work to study spruce budworm distribution in the crown as the infestation progresses, so No. 3 was easily the

logical choice. For the trees used in the population distribution study described above (Sec. 8.1), the branches were arbitrarily selected in the combination 2 : 2 : 1 : 1, that is, 2 from level *A*, 2 from *B*, 1 from *C*, and 1 from *D*. The percentage distribution of branch surface based on these sample branches was found to agree fairly well with the percentage distribution of branch surface for the whole crown. Accordingly, the combination 2 : 2 : 1 : 1 was tested on a broader basis in the field but was soon reduced to 1 : 1 : 1/2 : 1/2 for statistical reasons (Sec. 12.2). The latter combination, of course, provides the same relationship between levels but reduces the intensity of sampling by one-half. The results provided by this combination, as revealed by the percentage distribution of branch surface in the course of insect sampling, are summarized in Table III, which is intended for comparison with the second row of figures in Table II. The percentage distribution of branch surface in a 1 : 1 : 1/2 : 1/2 combination is slightly low in *A*, high in *B*, low in *C*, and high in *D*. However, for all plots (Table III, last row) no level shows a difference of over 2.5% from actual branch surface distribution in the crown (Table II). Also (Table III), it is clear that the same sample unit is suitable for all age-classes of balsam fir used in the study.

TABLE III

PERCENTAGE DISTRIBUTION OF BRANCH SURFACE FOR SAMPLE BRANCHES DRAWN FROM THE CROWNS OF BALSAM FIR IN THE COMBINATION: LEVEL *A*, 1 BRANCH; LEVEL *B*, 1 BRANCH; LEVEL *C*, ½ BRANCH; LEVEL *D*, ½ BRANCH

Age class	Plot No.	No. of sample units	*A* Apical ¼	*B* 2nd ¼	*C* 3rd ¼	*D* Basal ¼
35	G5	100	4.7 ± 0.3	21.4 ± 0.8	33.1 ± 0.8	40.8 ± 0.9
	G10	30	10.6 ± 1.2	20.8 ± 1.4	30.5 ± 1.5	38.1 ± 2.2
	K2	80	6.6 ± 0.4	23.5 ± 1.0	30.3 ± 0.8	39.6 ± 1.1
55	Q1	90	8.5 ± 0.4	24.6 ± 1.1	29.8 ± 0.9	37.1 ± 1.2
80	G2	100	7.6 ± 0.5	20.7 ± 0.7	32.9 ± 0.8	38.8 ± 1.0
	G8	80	7.1 ± 0.4	25.4 ± 1.0	27.9 ± 0.8	39.6 ± 1.1
105	G4	120	7.2 ± 0.4	22.2 ± 0.7	30.4 ± 0.7	40.2 ± 1.0
	G9	40	6.3 ± 0.4	21.6 ± 0.9	29.9 ± 1.1	42.2 ± 1.2
	K1	80	6.6 ± 0.4	22.2 ± 0.7	32.8 ± 1.0	38.4 ± 1.2
All		720	7.0	22.6	31.0	39.4

In an earlier section it was concluded that the whole branch, or its longitudinal one-half, was the smallest unit that should be used in absolute population studies, but this unit was not specifically designated as the sample unit. We are now in a position to define the *sample unit* as a group of branches collected in the combination 1 : 1 : 1/2 : 1/2 from the apical to the basal quarters of the crown. These sample units constitute the random elements in sampling, their variation being the basis of *sampling error*. When the

sample unit is referred to in the balance of the paper, it may be taken to mean this combination of branches. Individual branches constitute the sample unit in certain types of survey work where only one mid-crown branch per tree is collected: for present purposes, however, it will be better to refer to the individual branch as the *unit of collection.* It has also been shown that the sample unit may be drawn from any side of the tree, as long as all sides have an equal chance of selection. In actual practice the side used first is often the side where the canvas sheet can be spread on the ground most easily to receive the branches dropped by the pole operator. This is random selection in that it is in no way influenced by the insect population. As the same trees are used for successive sampling, sample units are eventually drawn from other sides.

The number of square feet of branch surface represented by the average sample unit varies from 10.0 to 12.8 in different plots, and averages 11.1. This represents approximately 2.5% of the total branch surface in a 6-in. tree (Fig. 16). A double sample unit (2 : 2 : 1 : 1) represents a 5% sample. Several successive sample units can be drawn from the tree, therefore, before the crown is sufficiently thinned to affect it appreciably as an insect habitat. We have generally used each group of population trees for a period of two years. With one larval, one pupal, and one egg sample per year, the total loss of foliage is only 15% and is scarcely noticeable from the ground. On plots where intermediate sampling is conducted for the purpose of determining larval trends, the trees are ordinarily used for only one season.

This section has been concerned with the problem of securing a sample from the host plant which will be representative of insect population on the whole plant. Undoubtedly, the same problem will occur in all forest insect population work, but in some cases the distribution of the insect may permit a more simple solution, as shown by Morris and Reeks (26) for the winter moth.

9. Representativeness in the Stand

The problem of securing representativeness in the stand is similar to the problem just discussed, except that here we are concerned with different stories of trees rather than different levels within the crowns of one story. This problem has already been touched upon (Sec. 6) but it is necessary to consider in more detail both the distribution of budworm population as between two different stories of the same stand, and the method of selecting sample trees in any one story.

9.1 Distribution of Population in a Two-storied Stand

This discussion is limited to two-storied stands because stands with more than two stories are not of common occurrence on the Green River Watershed. Studies of stand history in this area suggest a long succession of even-aged stands, consisting predominantly of balsam fir, with each stand owing its origin to the removal of the previous stand by some major forest influence such as a wind-storm or a budworm outbreak (18). Thus most stands are

characterized by a very even crown story, in which the great majority of the trees are equal co-dominants. Up to the age of about 60 years, these stands are generally so dense that there is little opportunity for the establishment of an understory. Beyond that age, the overstory gradually breaks up through the action of wind and disease in removing individual trees, and reproduction becomes established on the forest floor. The 80-year age class, which is well represented today, commonly has an understory of this advance reproduction varying in height from a few inches to several feet. The branch surface represented by the understory in such stands is generally less than 10% of the total branch surface per acre. It will be shown below that such stands may still be considered, without risk of serious error, as single-story stands for purposes of budworm population work.

In the 105-year age class, the situation is quite different. Stands of this age class were old enough in 1912 to suffer some mortality in the last budworm outbreak, but the degree of mortality varied widely in different stands. Some were left intact and now support only the understory established through the gradual disintegration of the overstory; others suffered complete mortality and this gave rise to the dense 35-year age class which is common on the Watershed today. Those which suffered intermediate degrees of mortality, resulting in a thinning of the overstory, are now characterized by a well-defined understory which may be 30 ft. or so in height. The proportion of the total branch surface per acre contained in this understory is very variable; on the plots with which we are here concerned it is roughly 25%.

Comparative sampling for the spruce budworm has been conducted on the overstory and the understory of the same stands, using two plots in the 80-year age class and two plots in the 105-year age class. As relative populations per 10 sq. ft. of branch surface in the two stories are so markedly influenced by infestation history, it will be informative to summarize the results verbally rather than in tabular form.

At very low populations (under five larvae or five egg masses per 10 sq. ft.) in the overstory, no budworm specimens were found in the understory. Probably a few occurred there but were not encountered in the samples at this low population level. At higher populations, but below the point where competition for food occurred at any crown level of overstory trees, population per 10 sq. ft. in the understory was 5% of that of the overstory. Results for different plots varied from 0% to 7.6% and as there were no significant differences associated with larvae, pupae, or eggs, the mean of 5% is based on all stages. When the infestation reached the stage where competition for food occurred in the overstory, many larvae dropped to the understory. This was first observed for late-instar larvae in the first year of fairly severe loss of new foliage in the overstory. In the second and third years of severe defoliation, extremely high larval populations exhausted the new foliage of the overstory very rapidly, so that dropping occurred in the early instars. In the third year of severe defoliation on Plot K1, which has a longer infestation history than the other plots, population on the understory was 45% of that

on the overstory for third-instar larvae and 61% for pupae. These comparisons are based on population per 10 sq. ft. of branch surface; as understory trees have fewer shoots per unit of branch surface, population intensity may have been about equal in the two stories. Because of their sparse growth, understory trees lose both their new and old foliage rapidly and soon fail to provide food for dropping larvae. Thus, in the fourth year of infestation on K1, population in the understory declined to 27% of the overstory population in the larval stage and 27% in the pupal stage; in the fifth year, 18% in the larval stage and 16% in the pupal stage. Throughout the five years of severe infestation on this plot (1950-1954), the moths retained a very marked preference for the overstory when depositing the eggs. In only one year did the population of eggs per 10 sq. ft. in the understory exceed 5% of that in the overstory, and that occurred in 1952 when unusually high moth activity and dispersal increased this figure to about 15%.

It is now possible to estimate the magnitude of the errors that would be involved if the understory were disregarded in population studies. Consider first an 80-year stand in which 5% of total branch surface is contained in the understory, and a moderate population level, with understory population per 10 sq. ft. only 5% of overstory population. Estimates of population per acre based on the overstory alone will be low by $(5 \times 5) \div [(5 \times 5) + (100 \times 95)] = 0.3\%$. Even in a severe infestation, when population per 10 sq. ft. in the understory may be 50% of that in the overstory, population per acre would be in error by only 4.8%. If the same two degrees of infestation are compared in a 105-year stand where the understory contributes 25% of total branch surfaces, the corresponding errors are 1.6% and 14.3%; and in a stand where the understory contributes 50% of total branch surface, they are 4.8% and 33.3%. The understory can be disregarded, therefore, only when its contribution to total branch surface per acre is rather small, or before the severe stage of infestation is reached.

Life tables will also reflect such errors during the years of severe infestation because different proportions of the three insect stages are found in the understory. To illustrate, let us assume that the basic unit, branch surface, is accepted as a reasonably good expression of absolute population over a limited number of years (Sec. 13), and that life tables for a given stand in which the understory contributes 25% of total branch surface are to be based on the overstory alone. Let us say that it is the third year of severe infestation, and population per 10 sq. ft. in the understory bears these relationships to overstory population: Eggs (previous year)—5%, third-instar larvae—45%, pupae—60%. Mean overstory population per 10 sq. ft. is 2800 eggs, 240 larvae, and 25 pupae. (These data are from K1, rounded off to two significant figures.) Thus the life table shows a mortality of 91.4% from eggs to larvae and 89.6% from larvae to pupae. By some simple calculations in arithmetic similar to those of the preceding paragraph, the corresponding mortalities based on both stories are 90.6% and 89.1%. In this illustration

the errors in the life table are not very serious, but they would become more so in stands where the understory contributes a larger share of the branch surface.

9.2 Selection of Trees in Each Story

The universe has been defined as a homogeneous forest stand as typified by a 25-acre study plot, and the decision has been reached that each crown story in the stand will be sampled as a separate stratum. According to the principles of random sampling, the trees used for population work should be selected from each story by random methods that ensure an equal chance of selection to every co-dominant tree on the 25 acres. Very probably this would be the most efficient design so far as sampling precision alone is concerned. However, a design with a small sampling error is preferable to one with a larger error only if the cost per sample unit is the same for both designs. What is wanted is the maximum precision of results per unit of cost. The consideration of costs, in terms of the number of man-days that can be assigned to the sampling work (Sec. 12.6), often leads to the selection of less random, and less precise, designs. The design adopted in the present study may be called the cluster design, although its objects are not exactly the same as those generally associated with cluster sampling (14). Cluster sampling involves the division of the population of elementary units under consideration (e.g., the trees on a 25-acre plot) into groups or clusters. In the present work, all trees in the cluster are sampled; in alternative designs, random subsamples are sometimes drawn from the cluster and this constitutes 'two-stage sampling'. Clustering has been used effectively to reduce costs (or man-days) in various types of sampling where the time involved in travelling from one elementary unit to another over a large area has been an important part of the cost function (14). It has been used frequently in forest insect sampling, although not referred to by name; the 'sampling points' used in extensive budworm survey work are essentially clusters of trees (23), but the results are plotted on a map instead of being treated as primary units for the estimation of total population over large areas.

The relation of the cluster design to the cost function will be discussed later (Sec. 12.6). Its value is directly associated with the mechanics of collecting foliage. Tree platforms and trestles lend themselves to cluster sampling and the time required to construct them for the sampling of individual trees would be prohibitive. The extension ladder also lends itself to cluster sampling, where it can be swung from one tree to another without lowering the extension or folding the brace. Even when the pole pruner can be used from the ground there would be a considerable wastage of time if the pruner had to be dismantled and all sampling equipment packed for a trip of several hundred yards through the stand to the next tree.

Ten trees per cluster have commonly been used in the Green River studies. In the population work to date, which in one sense has consisted largely of pilot sampling, replication of clusters in each stand has been limited by considerations of time to a few plots and to the larval stage of the insect.

The objects of the project have made it necessary to develop life tables in different stands during the early phases of the infestation, before sampling designs and techniques could be fully established. Developments planned for the immediate future include:

1. Greater replication of clusters for all plots and all stages of the insect. All 25-acre plots in the series have been divided into 2-chain grids and this will permit a random selection of cluster locations.

2. Frass-drop measurement under the population sampling trees of each cluster and under other clusters randomly dispersed in the stand. The comparison of frass-drop will provide a rapid means of assessing intercluster variance in larval population and of determining whether the clusters used for direct sampling are representative of the stand. In this application of frass measurement, which has already been tested on a limited scale in the present work, very simple traps are used and, as total seasonal frass-drop is not required, they need be left in each stand for only a day or two. It is assumed that all non-sampling errors, arising from frass retention in the feeding sites, the solubility factor, and wind dispersal, will have equal magnitude for all clusters of trees.

To date, the selection of the trees comprising each cluster has been a compromise between several factors, including a desire for central location in the stand, accessibility as provided by trails, and the suitability of the cluster with regard to the ease of ladder manipulation. As there is no reason to suspect that any of these factors are related to insect population, it seems safe to conclude that no great violence has been done to the principle of randomization. Also, with one or two exceptions, the clusters were selected before defoliation became very noticeable. Defoliation, or any similar effect of the insect on the plant, is commonly related to population, and when this stage of the infestation is reached it is important to select the sample trees by more objective and random methods in order to avoid any bias on the part of the selector. When mortality appears in the stand, dead trees constitute a further problem. This stage has just been reached on one of the Green River plots (K1) and it is proposed to select only living trees for continued sampling. Spring and fall timber cruises of the stand each year will provide up-to-date information on the proportion of dead trees in each diameter class, and the estimates of branch surface per acre can be revised accordingly (Sec. 13). This illustrates another advantage of the branch surface method; it permits the continued assessment of absolute population as the stand is decimated by the insect.

The preceding remarks apply particularly to one-story stands and to the overstory in two-story stands. In selecting sample trees from the understory, greater precautions must be used to ensure representativeness. High budworm populations on understory trees result largely from the dropping of larvae from the overstory. Thus, intertree variance in the understory is higher (Sec. 11) because population depends upon the position of the tree in relation to overstory trees. It is possible to find all ranges of defoliation from

100% immediately under large overstory trees, to less than 25% in openings where there is no overstory. Our understory samples to date are clustered, because they are obtained in the small section of the stand where the overstory is sampled, but their selection has been more systematic. A starting point is selected at random and a compass course is followed, with offsets to keep within the cluster area. Trees are selected for sampling at specified distances along the lines, regardless of their position in relation to overstory trees.

Sections 8 and 9 have been largely concerned with the development of a sampling design for intensive population studies on the spruce budworm. The design that has been chosen may be described very briefly:

1. *Proportionate sampling* by vertical levels within the crown of each tree.
2. *Stratified sampling* by crown stories within the stand (and sometimes by host species or flower production within stories; see Sec. 11).
3. *Cluster sampling* within strata.

10. Correlation Between Successive Samples

One of the reasons for developing a mechanical technique for sampling standing trees is to take advantage of the correlation that may exist when successive samples are drawn from the same trees. That such correlation would exist in spruce budworm populations was not known, of course, when the technique was developed; it was suspected, however, from the results of earlier work on both the larvae (19) and cocoons (29) of another defoliating insect.

In the Green River studies, sampling for the eggs, larvae (third instar), and pupae of each generation is conducted on the same 10, numbered trees of each cluster. This permits estimates of correlation in population between the different stages. The retention of most sampling clusters for two seasons also permits the estimation of correlation between the same stages in successive generations. For reasons presented below (Sec. 11), the original variates (insects per 10 sq. ft.) were subjected to the transformation $y = \log_{10}(x + 1)$. Correlations were then calculated by the product-moment method and the coefficients (r) were transformed to z values to permit tests of homogeneity, as described by Snedecor (32). As a result of these tests, it was found permissible to pool various estimates of r, as shown in Table IV. The correlation coefficients are not very high. In view of the low sampling intensity in each tree (Sec. 8.3) and the rather low populations (with their relatively high variances) on most of the plots during the period, this was not unexpected. However, the number of correlation tests is sufficient to permit significant conclusions concerning the nature of the correlations. These conclusions apply to overstory trees in stands of the four age classes used in the population work.

Considering first the different stages within one generation, there is evidence of positive correlation between eggs and larvae (E_xL_x in Table IV). Of the 14 coefficients, 79% are positive, whereas 50% would be expected in the absence of correlation, and the pooled value of r has some significance

TABLE IV

CORRELATION BETWEEN SUCCESSIVE SAMPLES FROM THE SAME TREES

(Each correlation is based on 10 pairs of overstory trees and the following symbols are used: E = eggs, L = larvae, P = pupae; x = one generation of the budworm, y = the generation following x; r = product-moment correlation coefficient)

Correlation between	No. of correlations	% positive correlations	Pooled value of r	Degrees of freedom
E_xL_x	14	79	+.23*	98
L_xP_x	37	89	+.39**	259
E_xP_x	14	54	+.08	98
P_xE_y	39	49	+.03	273
E_xE_y	14	93	+.36**	98
L_xL_y	19	63	+.20*	133
P_xP_y	14	50	+.06	98

* *Significant within the 0.05 level.*
** *Significant within the 0.01 level.*

($p < .05$). The correlation between larvae and pupae in the same generation (L_xP_x) is somewhat stronger. However, there appears to be little correlation between eggs and pupae of the same generation (E_xP_x), or between the pupae of one generation and the eggs of the following generation (P_xE_y). Considering the same stages of the insect in successive generations, E_xE_y shows the most significant correlation, L_xL_y shows definite evidence of correlation, while P_xP_y shows only questionable evidence.

To interpret these correlations biologically, it appears that ovipositing moths show a greater attraction to some trees than to others and that the preferred trees tend to have the highest egg populations year after year. Despite the dispersions that occur in the first and second instars (24), there is a tendency for the same trees to have the highest populations of third-instar larvae. By the time the pupal stage is reached, however, the correlation with egg population has largely disappeared. As a number of density-dependent mortality factors operate during the life cycle, it is reasonable to presume that the high population trees suffer greater relative reductions than the others, and that this causes the progressive reduction in correlation. Larval populations of the European spruce sawfly showed high correlations from year to year at high population densities, with a tendency for reduction of correlation following the action of a density-dependent larval disease (19). At endemic population levels of the spruce budworm, correlations may be difficult to demonstrate because of the relatively high sampling errors (Sec. 12.5). Also, although sufficient data are not yet available in the advanced stages of infestation, it is expected that correlations between successive generations may tend to disappear, or be reversed, as the preferred trees are stripped of foliage.

The demonstration of correlations supports the sampling design and shows that there is a statistical advantage in drawing successive samples from the

same numbered trees, at least under the conditions of a developing infestation. Statistical methods appropriate for paired variates (29, 32) will provide greater sensitivity in the detection of population changes, and life tables based on sampling data for the same trees throughout the insect generation will be stronger than those that are not. As the removal of too many branches may affect the tree as a population site, the same trees should not generally be retained for more than two generations of the insect. The best time to initiate sampling on a new cluster of trees is, of course, in the egg stage.

There is yet another way in which correlation affects the analysis of population data. The proportionate sample from each tree crown has been defined as the sample unit, and it has been indicated that the average size of the sample unit is about 11 sq. ft. (Sec. 8.3). Variations in the size of this unit occur from tree to tree and from one sampling date to another, depending upon the size of the branches that happen to be collected. Throughout the analysis, however, population per 10 sq. ft. for each sample unit has been given equal weight in the determination of plot means. Is this justified or should the tree estimates be weighted according to the number of square feet in the sample unit ? In the present section it has been learned that each tree in the stand has a certain status as a 'budworm producer' and that this status tends to be maintained from year to year. The status is determined by more obscure factors than branch size; in other words, population per 10 sq. ft. is not higher in a large sample unit than in a small one. Moreover, the tree is accepted as the major source of variance in the universe and plot means are really estimates of mean population per tree. It seems reasonable, therefore, that each tree should carry equal weight in this estimate, regardless of the fact that some trees happen to be represented by larger branches than others.

The presence of correlation may also help to explain the nature of frequency distributions. Population distributions for the spruce budworm, and for many other insects that have received detailed study, are of the negative binomial type (Sec. 11). If tree frequencies are plotted over budworm population density, the frequency curve is asymmetrical because it has a long 'tail' on the right; i.e., it has positive skewness. The negative binomial is a type of 'contagious' distribution which can arise through the effect of certain non-random elements on the distribution of the insect. In some cases contagion can be explained by the fact that the eggs are deposited in clusters, or the larvae tend to feed gregariously; so distribution is not random because the presence of one insect in the sample unit increases the chances of finding others. This simple explanation will hardly suffice for the spruce budworm. Although the eggs are laid in masses, dispersion in the first and second instars is generally rather effective in obscuring this source of contagion; moreover, the frequency distribution for egg masses, as well as for eggs, follows the negative binomial pattern. The same is true of larvae and pupae, although there is no tendency for the larvae to feed gregariously. The correlation between egg-mass populations in successive generations suggests that an alternative explanation may lie in the relative attractiveness of individual

trees to the insect. It is well known that insects can detect trees of the preferred host species through olfactory stimuli. It may be theorized that within the preferred host species, certain trees offer much stronger stimuli than the average tree and that the non-random nature of insect distributions is attributable to characteristics of the tree rather than the insect. The weak point in this theory, so far as the budworm is concerned, is that pupal populations conform to the negative binomial, although the correlation with egg populations (and hence with the original tree stimulus) appears to have largely disappeared by the time the pupal stage is reached. As a third possibility, the appearance of contagion could be inherent in certain sampling methods (5).

Resolution of these questions would be of interest biologically, and might also lead to improvements in sampling design. An adequate series of trees that can be assigned ranks according to budworm population are now available on different Green River plots. Experimental work is planned to ascertain whether the stimulus offered by the consistently high population trees is olfactory, or whether it can be explained by such factors as the exposure of the crown. Concurrently, frequency distributions for the budworm and for other defoliators of fir will be studied in more detail in an attempt to understand their biological significance.

11. Transformation of Data

In this section, and those immediately following, attention is directed from field techniques and designs to methods of analyzing the population data. In the development of the general sampling design, this subject must receive earlier attention, of course, than its listing here would indicate. Many of the decisions reached above (cf. Sec. 8.1) have been based on at least a preliminary examination of frequency distributions and appropriate transformation of data.

Statistical theory and many of the most useful procedures such as variance analyses and '*t*' tests are predicated on a frequency distribution of the normal, or Gaussian, type in which the variance is independent of the mean. As Finney (11) has pointed out, the name is unfortunate and must not be understood as meaning that it is a standard type, deviations from which represent abnormality. Other distributions should be called anormal, not abnormal. For many types of biological data, and particularly for insect population samples based on units of space or time, the negative binomial distribution may be more frequently encountered than the normal (1, 5). To ensure valid statistical analyses, it is necessary to transform such data to a new scale which makes the variance independent of the mean. For distributions of the negative binomial type, transformation of the original variates to logarithms generally has the desired effect of stabilizing variance.

The most direct way to ascertain the nature of the frequency distributions for a given insect species is to examine a large number of sample units (preferably several hundred) collected at the same time and place. The results can be listed in a frequency table and compared directly, by chi-square tests, to

the theoretical frequencies that would be expected when different types of distributions are assumed. When such large-scale sampling at one time and place is not practical, the nature of the distributions can generally be deduced from the relationship between variance and mean, as based on a number of smaller samples collected on different plots and dates. The mean ($\bar{x}$) and variance (s^2) were calculated for each cluster of 10 trees sampled on the various plots of the Green River Watershed during the past five years. The results are based on 54 points for egg masses (Fig. 9) and 119 for larvae and pupae (Fig. 10). Larvae and pupae were first plotted separately but as they showed identical relationships they were combined. Egg masses are treated separately because they tend to show a higher variance for a given mean than do larvae and pupae. The range of means represented in this work is as great as any that is likely to be encountered during spruce budworm infestations; certain plots outside the main infestation area supported only a low population during the period while on others the infestation reached a peak and the population started to decline as a result of food depletion.

From the general trend of the plotted points (Figs. 9, 10) we can at once rule out the possibility of the normal distribution (s^2 independent of $\bar{x}$), the poisson ($s^2 = \bar{x}$), as well as any distribution that implies a linear relationship ($s^2 = k\bar{x}$). The curves plotted in the graphs ($s^2 = \bar{x} + \bar{x}^2/k$) represent the relationship to be expected between s^2 and $\bar{x}$ for the negative binomial distribution. These curves have not been fitted mathematically; the values for k are

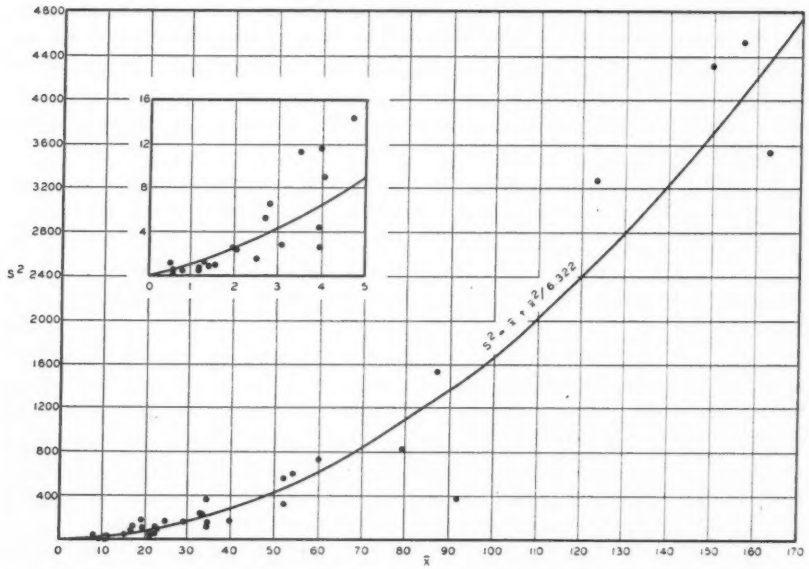


FIG. 9. Relationship between variance and mean for spruce budworm egg-mass populations on standing trees, 1950-54. Each point plotted is based on a cluster of 10 trees. s^2 = intertree variance; $\bar{x}$ = mean number of egg masses per 10 sq. ft. of branch surface. Inset shows points below $\bar{x} = 5$ on an expanded scale.

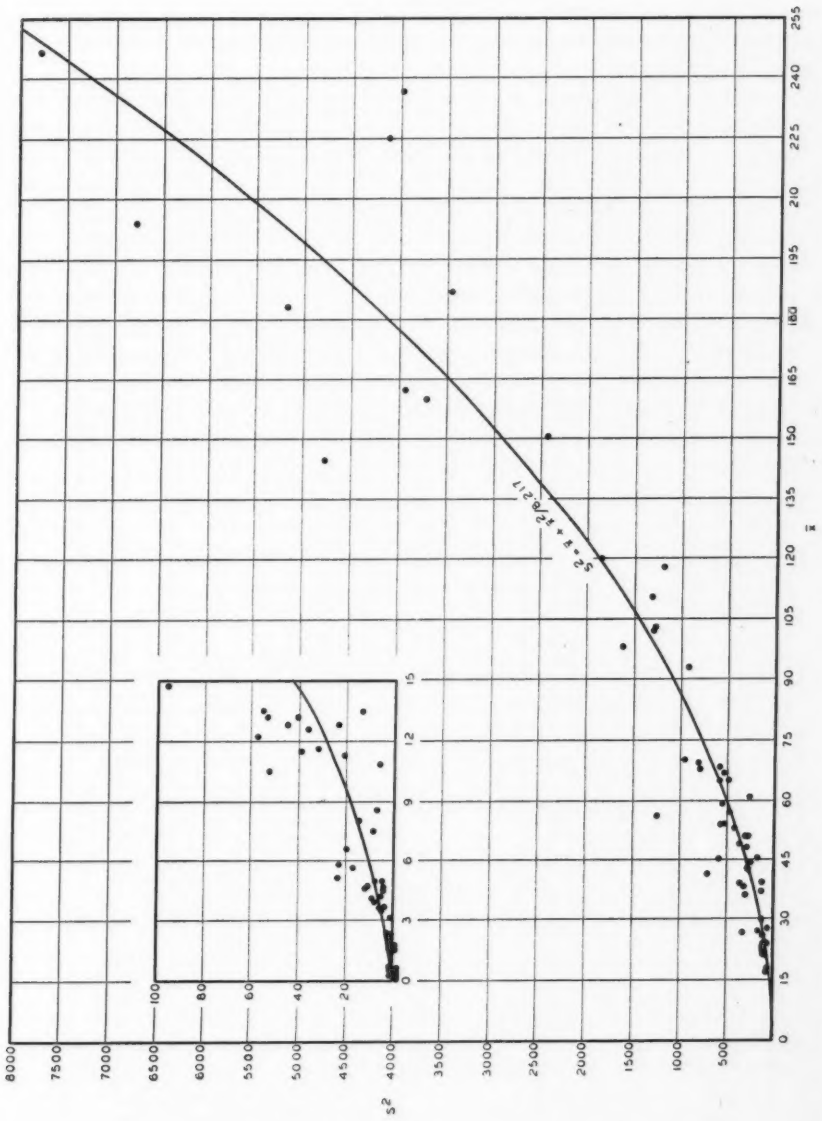


FIG. 10. (Read broadside.) Relationship between variance and mean for spruce budworm larval and pupal populations on standing trees, 1950–54. Each point plotted is based on a cluster of 10 trees. s^2 = intertree variance; $\bar{x}$ = mean number of larvae or pupae per 10 sq. ft. of branch surface.

only first approximations obtained by summation, $k = \Sigma\bar{x}^2/(\Sigma s^2 - \Sigma\bar{x})$. Even so, the curves appear to fit the plotted points very well and it seems safe to conclude that the population data are adequately represented by the negative binomial distribution.

To test the effectiveness of a logarithmic transformation in removing the relationship between mean and variance, the same data have been replotted following the transformation of the original variates in each cluster (Figs. 11, 12). The transformation was of the form $y = \log_{10}(x + 1)$. The figure '1' in the formula serves to simplify calculations by eliminating negative logarithms and helps to stabilize variance at low levels of population. The scatter of points that now results suggests no clear relationship between s^2 and $\bar{y}$, so the transformation seems appropriate for the data. The transformation suggested by Beall (3) for entomological data, of the form $y = k^{-\frac{1}{2}}\sinh^{-1}(kx)^{\frac{1}{2}}$, has also been tested in a preliminary way on some of the same data and appears to be equally satisfactory. However, the log transformation has been used more commonly in the present work because it permits of more simple manipulation in calculating optimum sample size, etc. (Sec. 12).

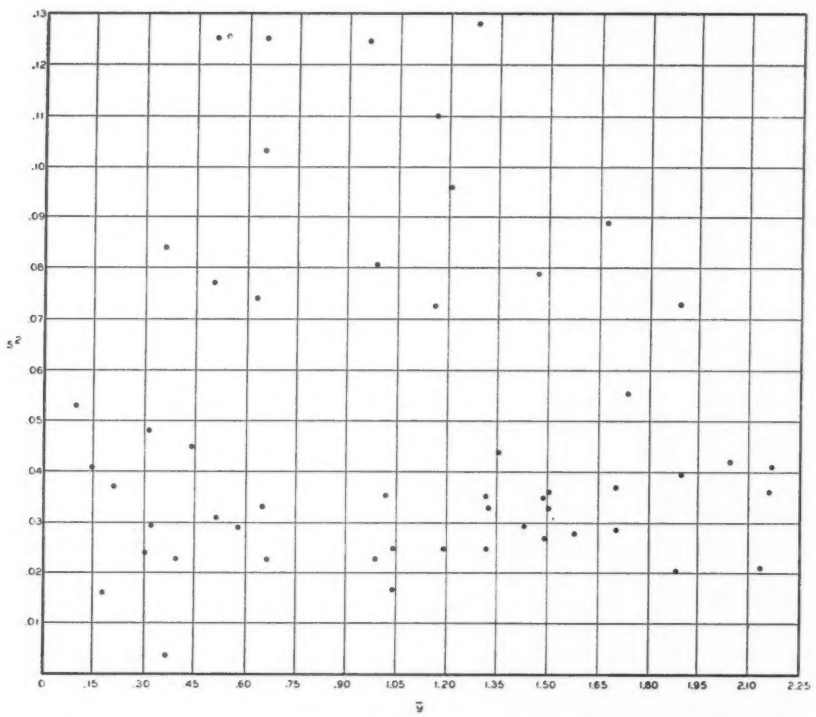


FIG. 11. Variance (s^2) plotted over mean ($\bar{y}$) for egg-mass population data (Fig. 9) following transformation of the form $y = \log(x + 1)$.

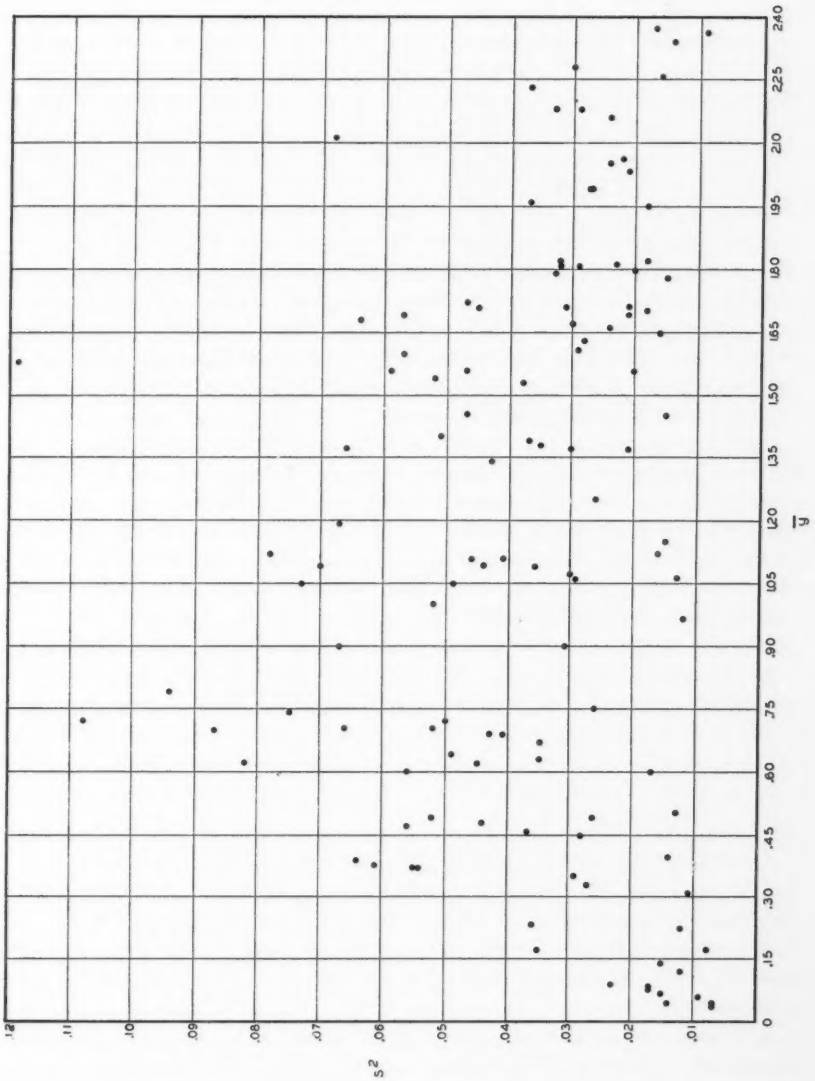


FIG. 12. (Read broadside.) Variance (s^2) plotted over mean ($\bar{y}$) for larval and pupal population data (Fig. 10) following transformation of the form $y = \log(x + 1)$.

The estimates of variance for a given level of $\bar{y}$ show a fairly broad scatter (Figs. 11, 12). This is to be expected because each estimate is based on a cluster of only 10 trees. However, if it is accepted that s^2 is independent of $\bar{y}$, and is homogeneous from stand to stand, there is no objection to pooling these estimates to obtain a single, stronger estimate. As all points have equal weight, a simple arithmetic mean may be used. The pooled estimate of variance for egg masses is 0.0518 and for larvae and pupae, 0.0365. These estimates will be strengthened, of course, as more data accrue from further sampling. Also, as further data accrue, more refined calculations of k will be made (1, 5), and the efficiency of other transformations will be compared. Anscombe (1) suggests a transformation of the form $y = \log(x + k/2)$.

No formal tests for the homogeneity of variances have been applied to the data on hand. However, the plotting of variance over mean was first done on a large sheet where all points could be labelled according to plot number and year, and certain points were discarded before the final graphs (Figs. 9–12) were prepared. The data that were discarded showed consistently high variances for one of the following reasons:

1. Flowering habits of the stand.—Stands in the 35-year age class show a relatively light production of staminate flowers, largely confined to the more open, dominant trees, while stands in the 80- and 105-year age classes show heavy flower production on all co-dominant trees. At intermediate age classes, such as the 55-year class used in the present work, flower production on co-dominants may range from zero to fairly heavy, depending on the exposure of the individual trees. As flowering trees bear higher larval populations, the population variance in such stands may be higher than the average if no attention is paid to this factor in selecting the sample trees. One such stand (Q1) sampled in the flowering year 1950 had a variance of 0.1276 for larvae and 0.0825 for pupae, as compared with the average of 0.0365. Subsequently, in intermediate age classes, the sampling design and analysis were adapted to recognize flowering and non-flowering sub-strata within the main stratum or story.

2. Overstory in advanced stage of infestation.—Plot K1, where mature trees started to die in 1954, showed variances of 0.0773 for larvae, 0.0644 for pupae, and 0.1718 for egg masses. This suggests that variance may increase when an advanced stage of infestation is reached, so these results have been held separate from the main body of data pending further sampling in advanced infestations.

3. Understory in advanced stage of infestation.—In the early stages of infestation, before the understory is affected by the dropping of larvae, variance appears to be similar to that in the overstory. In later stages, for reasons discussed (Sec. 9.1), variance in the larval and pupal stages becomes high in the understory. A pooled estimate for larvae and pupae in the years of advanced infestation on K1 is 0.1512, as compared with the normal of 0.0365.

With these exceptions, for which logical explanations can be advanced, the original plotting revealed no consistent tendency for one plot to have a population variance significantly higher than another. This tentative conclusion will be subjected to more objective statistical tests as further data are secured.

Finney (10) has shown that estimates of variance based on the original values may be very inefficient. Clearly, then, the spruce budworm population data should be transformed to logarithms in any computations involving the variance. Transformation also has a considerable advantage in permitting the use of a pooled estimate of variance in place of the rather weak estimate based on an individual cluster of trees. This naturally introduces the question of how the mean should be expressed in the final presentation of results. Once the logarithmic transformation has been made, and found appropriate, there is no statistical advantage in transforming back to the terms of the original variates; the data from a given sample can best be summarized as to central tendency and dispersion by giving the mean and the variance of the logarithms. This will provide consistent and efficient estimates of the parameters of the entire 'population of logarithms' and, for most purposes, will be quite satisfactory. For the purposes of life tables, however, it has a number of practical disadvantages. Life tables are designed to show not only the population density (l_x), but the mortality (d_x) and the rate of mortality ($100\ q_x$) during short age intervals (x) of the insect (24). The use of logarithms in the l_x column, and, consequently, in the d_x column, would surely give rise to complications in computing $100\ q_x$. The rate of mortality is the most important part of the life table and should be readily interpretable by the average reader who wants to compare mortalities between different age intervals and different life tables. The calculation of population per acre (Sec. 13) and population intensity (Sec. 14.1) would also be considerably complicated. For these purposes it seems advisable, at least at the present writing, to present the population means in terms of the original variates, i.e., numbers of insects. The antilog mean (in the present case, antilog $\bar{y} - 1$), is an estimate of the geometric mean (only approximately, in the present case, because of the constant '1' in the transformation) and is generally more appropriate than the arithmetic mean as a measure of central tendency in markedly skewed distributions. However, the geometric mean is always lower than the arithmetic mean, unless all variates happen to be equal; for the plots included in Fig. 9, the geometric mean averages 87% of the arithmetic mean, and for those in Fig. 10, 90%. In estimates of population per acre and of changes in absolute population, use of the geometric mean would therefore give biased results, particularly if the distributions should become more skewed in advanced stages of infestation. The use of the median would be attended by the same difficulty.

For these reasons the arithmetic mean, as determined from the original variates, has been used to date in the present study for the preparation of life tables and for estimates of population per acre or per unit of food. The

arithmetic mean provides a consistent estimate of the population mean, but the estimate is not of full efficiency when the distribution is skewed. This is discussed by Finney (10), who presents formulae for obtaining efficient estimates of the mean and variance, in terms of the original variates, when the logarithms of the observations are normally distributed. The efficiency of the mean depends upon the magnitude of the variance on the logarithmic scale. When the variance of the natural logarithms is 0.69 (variance of common logarithms 0.13), the efficiency of the mean is 93% or better (10). At this level of variance, corresponding to a coefficient of variation of roughly 100% in the original population, Finney concludes that the mean will be satisfactorily estimated by the direct sample mean, and that there is little advantage in estimating a corrected mean. In applying his formula to actual data, which have a coefficient of variation of 75%, he finds an efficiency of 97% for the mean. For spruce budworm populations, the pooled variance of the common logarithms is only 0.0365 for larvae and pupae and 0.0518 for egg masses, and the coefficient of variation of the original variates is generally less than 75%, except at very low populations. So it may be concluded that the efficiency of the direct sample mean is rather high, and is certainly well within the range of other errors arising from either sampling or non-sampling sources.

Although the mean was estimated from the original variates with efficiencies of 93% and 97% in the situations described above, the variance was estimated with efficiencies of only 28% and 45%. Thus the decision to base budworm population means on the original data and to base variance on the transformed data appears to be sound.

12. Optimum Sample Size

This section is concerned with the following questions: (1) What degree of precision is required in intensive sampling where the object is the development of life tables? (2) What is the optimum sample size in terms of the numbers of sample units per tree, trees per cluster in each stratum, and clusters per plot? (3) How are optimum sample size and design affected by changes in population density? (4) What is the total cost function per plot, or stand, and how many plots can be studied with a field establishment of fixed size? These questions are of primary importance and must be answered before logical decisions can be made as to the most useful and economic allocation of sampling resources. The answers will also influence the very basic decision as to whether or not an intensive sampling program for a forest insect is justifiable or necessary in a given situation. Despite their prime importance, neither the problems themselves nor even the compilation of cost functions have received much attention in published works on forest insect sampling.

Optimum sample size may be readily estimated with the aid of available statistical techniques after suitable pilot sampling has been done. The techniques vary according to sampling design and are generally based on the assumption of either a fixed degree of precision or a fixed cost. In forest insect

sampling, neither of these two limiting factors can be fixed very rigidly on the basis of the limited experience to date. The order of approach in the paragraphs that follow will be: (1) assumption of a fixed precision; (2) calculation of the optimum numbers of sample units, trees, and clusters per plot required to provide this precision; (3) calculation of cost function per plot; (4) estimation of the number of workers needed to develop life tables for a representative series of plots. Throughout this discussion, the word 'cost' is used in a very limited sense to represent the man-hours of work involved in collecting and examining the foliage samples during the field season. It is essentially this aspect of the total cost function that limits the amount of population sampling that can be done each year. No consideration will be given to the costs involved in overhead, provisioning of field parties, analyzing of population data, or similar non-limiting factors.

12.1 Degree of Precision Required

Of the various problems listed above, the one concerning the degree of precision required is by far the most difficult and can be solved only on a very arbitrary basis at the present writing. Insufficient work has been done on forest insect populations to enable us to say, on the basis of experience or published data, what degree of precision is necessary to permit the solution of a given type of problem. This is particularly true when detailed life-table development is the object of the sampling, as it now is for the first time. Many factors must be taken into consideration, not all of which lend themselves to direct mathematical treatment. The more important ones are discussed below:

1. We are concerned, first of all, with the definition of population means for the whole plot with reasonably good precision. This is so because we wish to reach conclusions that apply to the plot, or stand, rather than conclusions that must be limited to a small cluster of sample trees. It is so because we also wish to express population on the absolute basis of land area and to use it in conjunction with bird and mammal populations as obtained on the 25-acre plot. Therefore the standard error of the mean ($s_{\bar{x}}$) for the sample trees must represent an acceptably small percentage of the mean ($\bar{x}$).

2. In life-table studies we are also concerned with the definition of mortality between successive stages of the insect; that is, we are concerned with the standard errors of mean differences, which may be represented as

$$s_{(\bar{x}-\bar{y})} = \sqrt{s_{\bar{x}}^2 + s_{\bar{y}}^2 - 2r(s_{\bar{x}}s_{\bar{y}})}$$

where $s_{\bar{x}}$ applies to the larval stage and $s_{\bar{y}}$ to the pupal stage, for example, and r is the coefficient of correlation between the successive samples. For forest insect populations, $\bar{x} - \bar{y}$ is generally greater than $\bar{y}$ (see No. 6 below); so perhaps it is safe to assume that if $s_{\bar{y}}$ represents an acceptably small percentage of $\bar{y}$ then $s_{(\bar{x}-\bar{y})}$ will represent an acceptably small percentage of $\bar{x} - \bar{y}$, particularly when $s_{(\bar{x}-\bar{y})}$ is significantly reduced by r. By using the pooled variance of 0.0365 given above for larvae and pupae, and the r value of + .39

from Table IV, it may be readily demonstrated that $s_{(\bar{x}-\bar{y})}$ is reduced by 21% through the presence of correlation. We are also concerned with $s_{(\bar{x}-\bar{y})}$ in making comparisons between different plots or different generations on the same plot.

3. In life-table studies designed to provide information on the effect of forest management on insect epidemiology, we are particularly concerned about the number and variety of forest conditions that can be included in the population work. The objects of such a project may be defeated by setting such a high degree of precision that only one or two conditions can be studied with the assigned field establishment. At the same time, no useful purpose is served by securing data of doubtful statistical significance in a large number of forest conditions. In reaching a suitable compromise between these two extremes, personal judgment must play a leading role until the path is lighted by additional data and experience.

4. It is unlikely that serious conclusions concerning epidemiology and forest management can be formulated after one season's work. One of the objectives already defined (24) is the preparation of continuous life tables for successive generations of the insect in the selected stands. Thus replication is achieved in time as well as in place. The effect of the replication in time is difficult to evaluate statistically. Population figures for a given plot cannot be directly pooled from year to year, of course, because they generally represent different levels of population. However, mortality rates, or ratios of increase or decrease between specific periods, may often be pooled. In this regard, differences between logarithmic means will undoubtedly be useful both in pooling and in variance analyses, because they represent the logarithms of the corresponding ratios. The availability of an intergeneration variance as a major source of variance for the testing of broad conclusions adds weight to those conclusions; it also encourages us to accept a lower degree of precision for each individual population estimate than would be acceptable if there were no replication in time.

5. The accuracy of population work, as limited by the retention factor and by human error, has been discussed in earlier sections. (*Accuracy* and *precision* are used in the sense defined by Finney (11); the former is conditioned by non-sampling errors and the latter by sampling errors.) Although the non-sampling errors in spruce budworm counts cannot be evaluated exactly, it seems reasonable to expect that they will reach 10% under certain conditions, even when every precaution is taken to minimize them. There is no point in striving for a degree of precision so fine that it is out of proportion to the accuracy of the counts.

6. In comparison with most other organisms, insects have a high fecundity and a high rate of mortality. Natural populations are therefore very dynamic, particularly during the course of an epidemic. For example, spruce budworm populations may increase at the rate of eightfold per year when an epidemic is developing (Sec. 12.5). Larval population at the peak of an epidemic is at least 5000 times higher than the population between outbreaks. The

average female lays 150 eggs, and a mortality rate of 98.7% is necessary in each generation to prevent population increase. Insect population work is therefore generally concerned with the appraisal of large differences, rather than the detection of changes that are of small magnitude in relation to mean population level. For this reason, experience may show that we can accept a relatively low degree of precision, of an order that might be quite unacceptable in laboratory experiments or in natural population work on larger organisms.

In the calculations that follow, the required standard error is generally set at 10% of the mean. The assumption that this degree of precision is required is arbitrary and, in view of the factors discussed above, further developments may well prove it to be either too low or too high.

12.2 Number of Sample Units per Tree

Is it better to draw one sample unit from each of many trees, or several sample units from each of a smaller number of trees ? The answer to this lies in the relationship between the components of variance associated with trees and with sample units, and in the cost of moving from tree to tree as compared to the cost of drawing and examining each sample unit.

It has already been shown that intertree variance is significantly higher than the variance between quadrants (and hence between sample units) within trees. In other words, if s_t^2 is the variance component for trees and s_s^2 is the component for sample units within trees, we know that $(N_s s_t^2 + s_s^2)/s_s^2$ is significantly greater than unity. To secure an estimate of s_s^2 based on more extensive data than the earlier variance study, two sample units per tree were drawn from each of the 10 trees in 18 of the regular sampling clusters. The 18 clusters were distributed in different stand types and represented different population levels from moderately low to very high. Sampling was conducted in the egg, larval, and pupal stages and the original variates (insects per 10 sq. ft.) in each cluster were transformed to logarithms and analyzed for inter- and intra-tree variance. 'F' varied between 2 and 12 and the mean value of s_s^2 was 0.0180 for egg masses, 0.0193 for larvae, and 0.0142 for pupae. As differences between stages were not significant, the three stages were pooled to give $s_s^2 = 0.0170$. Our strongest estimates of intertree variance are based on extensive sampling that employed one sample unit per tree (Sec. 11). So we may now write

	Egg masses	*Larvae and pupae*
Intertree variance ($s_t^2 + s_s^2$)	0.0518	0.0365
s_s^2	0.0170	0.0170
s_t^2	0.0348	0.0195

In view of what has been said in reference to the correlation between successive egg samples (Sec. 10), it is interesting to note here that the higher variance associated with egg sampling appears to result entirely from the component s_t^2.

In order to use these components in finding combinations of N_t (No. of trees per cluster) and N_s (No. of sample units per tree) that will provide equal sampling precision, we must next determine the value of the standard error for the required degree of precision. This is somewhat complicated by the fact that we wish to use means based on the scale of the original variates in conjunction with variance components based on the logarithmic scale. To illustrate, let:

$\bar{x}$ = population mean on original scale = 25 egg masses per 10 sq. ft.,
$s_{\bar{x}}$ = required standard error on original scale = 10% of $\bar{x}$ = 2.5,
$\bar{y}$ = population mean on log scale, where $y = \log_{10}(x + 1)$,
$s_{\bar{y}}$ = standard error on log scale,
then: $\bar{x} + s_{\bar{x}} = 27.5$; $\bar{x} - s_{\bar{x}} = 22.5$.

It has been shown (Sec. 11) that antilog or geometric means based on egg-mass populations are 87% of the corresponding arithmetic means. Although it will scarcely affect the results we may apply this correction before transforming so that

antilog $\bar{y} - 1 = 21.8$, antilog $(\bar{y} + s_{\bar{y}}) - 1 = 23.9$, antilog $(\bar{y} - s_{\bar{y}}) - 1 = 19.6$;
therefore $\bar{y} = 1.3579$, $\bar{y} + s_{\bar{y}} = 1.3962$, $\bar{y} - s_{\bar{y}} = 1.3139$.

This provides two values for $s_{\bar{y}}$, $1.3962 - 1.3579 = 0.0383$ and $1.3579 - 1.3139 = 0.0440$, because $s_{\bar{y}}$ is not symmetrical with respect to the mean. These two values may be averaged, but in the following calculations the smaller one has been used throughout because it provides larger, and therefore safer, estimates of N_t. In brief, then, we have specified the condition that the required standard error on the transformed scale must correspond to 10% of the mean on the original scale. That is,

$$s_{\bar{y}} = \log\left[\frac{(1 + 10/100)\bar{x} + 1}{\bar{x} + 1}\right]$$

where $\bar{x}$ is first reduced to correspond more closely to its antilog counterpart.

The equality

$$s_{\bar{y}} = \sqrt{\frac{N_s s_t^2 + s_s^2}{N_s N_t}}$$

may now be solved for N_t and the appropriate values substituted. Thus, for egg-mass populations,

$$N_t = \frac{0.0348\, N_s + 0.0170}{(0.0383)^2\, N_s}.$$

Thus, if $N_s = 1$, $N_t = 35$; and if N_s is increased to 2, N_t is decreased to 29, and so on.

In Table V, N_s and N_t are listed for four widely different levels of $\bar{x}$, as well as $N_s N_t$, which represents the total number of sample units that must be examined for each combination. As more than one sample unit is drawn from the tree, it is seen that N_t decreases only slowly and soon becomes practically constant. The total work, as represented by $N_s N_t$, increases at a much faster

rate than N_t decreases. It may be concluded that there is little advantage in drawing more than one sample unit per tree, unless the time required to move from tree to tree is very high in relation to the time required to draw and examine one sample unit.

TABLE V

COMBINATIONS OF N_s (SAMPLE UNITS PER TREE) AND N_t (TREES PER CLUSTER) THAT WILL PROVIDE EQUAL SAMPLING PRECISION

(The data apply to populations of the spruce budworm and to four levels of mean population ($\bar{x}$). The standard error is set at 10% of the mean)

	$\bar{x} = 1$		$\bar{x} = 5$		$\bar{x} = 25$		$\bar{x} = 125$	
N_s	N_t	N_sN_t	N_t	N_sN_t	N_t	N_sN_t	N_t	N_sN_t
Egg masses								
1	123	123	46	46	35	35	31	31
2	103	206	39	78	29	58	26	52
3	96	288	36	108	28	84	24	72
4	93	372	35	140	27	108	23	92
5	91	455	34	170	26	130	23	115
Larvae and pupae								
1	87	87	32	32	25	25	22	22
2	67	134	25	50	19	38	17	34
3	60	180	22	66	17	51	15	45
4	56	224	21	84	16	64	14	56
5	54	270	20	100	16	80	14	70

To test the last possibility, we may use a relationship based on calculus (14):

$$\text{optimum } N_s = \sqrt{k\frac{s_s^2}{s_t^2}}$$

where, in the present case,

$$k = \frac{\text{cost of moving from one tree to another}}{\text{cost of drawing and examining one sample unit}} = \frac{C_3}{C_4}.$$

It will be shown below (Sec. 12.6) that C_3 is 1.25 man-hours, as based on the use of the sampling ladder in one cluster of trees, and C_4 is 7.07 man-hours for egg sampling and about two man-hours for larval and pupal sampling. Thus, for egg sampling,

$$\text{optimum } N_s = \sqrt{\frac{1.25}{7.07} \times \frac{0.0170}{0.0348}} = 0.3$$

and for larval and pupal sampling, optimum $N_s = 0.7$.

If a random rather than a cluster sampling design is employed, C_3 is increased to 2.50, but optimum N_s is still less than one for egg masses and about one for larvae and pupae.

These results naturally pose the question whether sampling efficiency could not be improved by using less than one sample unit per tree. However,

where it is desired to maintain representativeness in the crown and to measure correlation between successive samples, it is difficult to see how the branch proportions 1 : 1 : 1/2 : 1/2 in one sample unit could be much reduced.

To summarize, the optimum number of sample units appears to be one per tree, considering the purposes of the present investigation. Probably for forest insects in general, any sampling design will be poor that requires the examination of all the foliage on the tree, or a large proportion of the foliage. For less exacting purposes, such as insect survey or the biological assay of a single stimulus, the use of a smaller sample unit such as one branch per tree is to be recommended. A smaller sample unit, necessitating a sacrifice of representativeness in the crown, may also be advisable for all types of investigations at low levels of population (Sec. 12.5).

12.3 Number of Trees per Plot

Techniques are available for cluster sampling designs (14) which can be used to find the optimum combination of trees per cluster and clusters per plot. However, as the available data suggest that intercluster variance is unlikely to be significant (Sec. 12.4), only the problem of finding the number of trees required to define mean population will be considered here. In other words, it is assumed that it will be found permissible to pool the data from the trees in all clusters on one plot.

Before calculating the number of trees required for a fixed degree of precision, it may be of interest to see how closely mean population at a cluster locus has been defined by the 10 trees in each cluster. Here again it is necessary to use original scale means in conjunction with logarithmic variances. The example that follows employs the same symbols defined above:

Let $\bar{x} = 50$ larvae per 10 sq. ft.; then the best estimate of the corresponding antilog mean is 90% of $\bar{x}$, or 45 (Sec. 11). Therefore $\bar{y} = \log(45 + 1) = 1.6628$. The strongest estimate of s_y^2 is 0.0365 (Sec. 11); therefore $s_{\bar{y}} = \sqrt{0.9365/10} = 0.0604$. Thus, $\bar{y} + s_{\bar{y}} = 1.7232$; $\bar{y} - s_{\bar{y}} = 1.6024$ and, antilog $(\bar{y} + s_{\bar{y}}) - 1 = 51.87$; antilog $(\bar{y} - s_{\bar{y}}) - 1 = 40.03$. These last two figures may easily be converted to the original arithmetic scale and compared to the mean of 50. It is simpler, however, and gives the same results to compare them to the mean of 45, and this gives 6.87 and 5.03 as the two estimates of the standard error. For our present purposes it is safer to use the larger estimate, so the standard error is $100(6.87/45) = 15.3\%$ of the mean.

If similar calculations are made for selected values of the mean, a curve can be drafted to show the relationship between the standard error and the mean. This has been done for larvae and pupae and for egg masses (Fig. 13, solid lines). The egg-mass curve is higher, of course, because of the higher variance associated with egg-mass sampling. At moderate to high levels of population, however, the mean has been defined surprisingly well by a sample of only 10 trees. As a matter of interest, corresponding curves have been plotted based on the variance of the original data without transformation (Fig. 13, broken lines). For selected levels of $\bar{x}$, $s_{\bar{x}} = \sqrt{s^2/10}$, where s^2 was

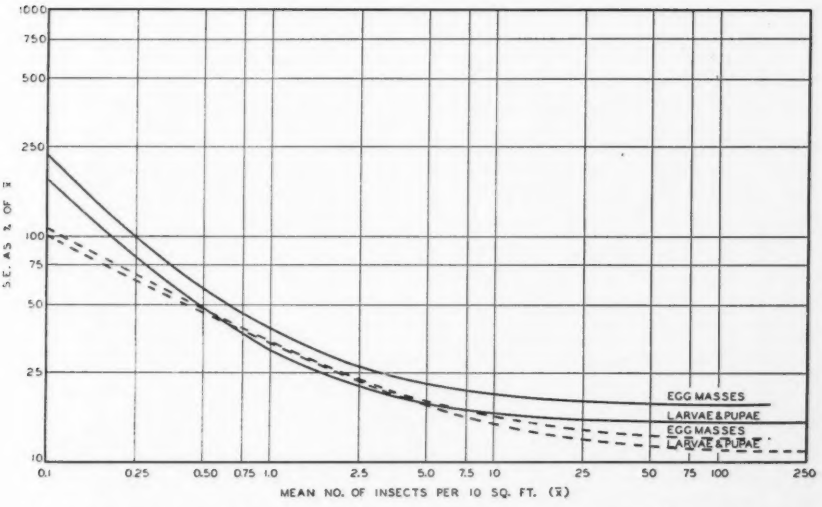


FIG. 13. The degree of precision with which mean population is defined by the 10 trees in one sampling cluster. The standard error (S.E.) is expressed as a percentage of the mean and plotted over mean population per 10 sq. ft. ($\bar{x}$) for egg masses and for larvae or pupae. Solid lines are based on the variance of transformed data; broken lines on variance before transformation.

read from the curves in Figs. 9 and 10. As the broken lines are generally lower than the solid ones, it is clear that variance would be underestimated if no transformation were applied.

The number of trees required to provide $s_{\bar{x}} = 10\%$ of $\bar{x}$ can be calculated by the same procedure illustrated in Section 12.2. As we are now interested in only one sample unit per tree, however, the formula for N_t simplifies to $N_t = 0.0518/s_{\bar{y}}^2$ for egg masses and $N_t = 0.0365/s_{\bar{y}}^2$ for larvae and pupae. The value of $s_{\bar{y}}$ for each selected level of $\bar{x}$ is determined by finding $\bar{x} + s_{\bar{x}}$ and transforming to $\bar{y} + s_{\bar{y}}$ (Sec. 12.2). Values of N_t have been plotted over the whole range of $\bar{x}$ (Fig. 14, solid lines) in contrast to the values obtained by using the original variance (Fig. 14, broken lines). In deriving the broken lines, $N_t = s^2/s_{\bar{x}}^2$, where s^2 is read from the curves in Figs. 9 and 10 and $s_{\bar{x}}$ is set at 10% of $\bar{x}$. When variance is based on the transformed data, the estimates of required N_t are generally higher. If the logarithmic transformation tends to 'over-correct', i.e., to reduce variance disproportionately at the higher levels of the mean, then the best estimate of N_t lies somewhere between the two extremes represented by the solid and broken lines in Fig. 14. There is some suggestion of overcorrection between moderate and high levels of population, especially for the larval and pupal data (Fig. 12), but not between low and moderate levels.

The method used here for computing the required N_t is generally applied to large-sample techniques, and the reader may wonder why methods more applicable to small samples have not been used. As the degree of precision

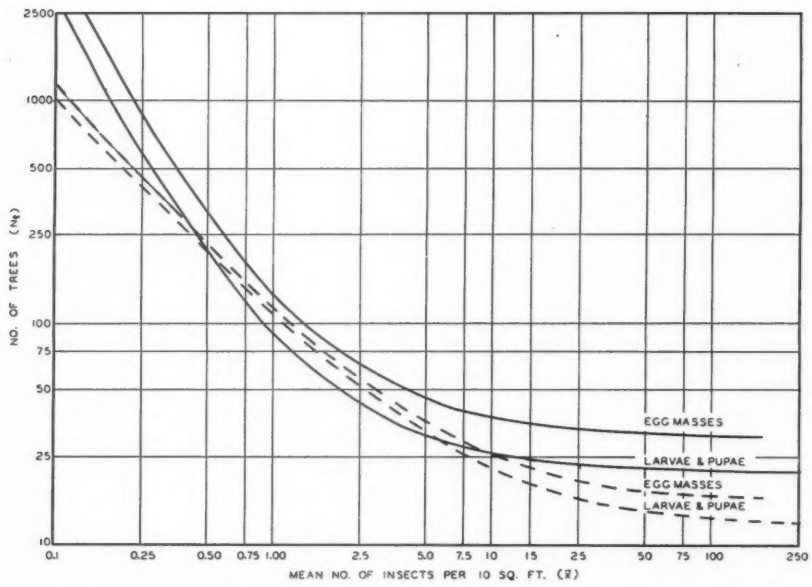


FIG. 14. The number of trees (N_t) required to define the population mean with a precision of S.E. = 10% of $\bar{x}$, where S.E. is the standard error and $\bar{x}$ = mean population per 10 sq. ft. Solid lines are based on the variance of transformed data; broken lines on variance before transformation.

required in life-table work is not yet known, it is felt that the simpler method associated with large-sample theory is sufficiently accurate for present purposes. In any case, as the smallest sample numbers required are 31 for egg masses and 22 for larvae and pupae (Fig. 14), the two methods will give rather similar results. For example, Snedecor's formula for sample size (32) can be adjusted as follows to permit the use of variance on the logarithmic scale (31):

$$N_t = \frac{(t_{.05})^2 s_y^2}{\left[\log \frac{(1 + p/100)\bar{x} + 1}{\bar{x} + 1}\right]^2}.$$

If $\bar{x} = 170$ egg masses per 10 sq. ft., $t_{.05} = 2.04$, and the required precision is $p = \pm 20\%$, then

$$N_t = \frac{(2.04)^2\ (0.0518)}{\left[\log \frac{(1.2 \times 170) + 1}{170 + 1}\right]^2} = 35,$$

as compared to $N_t = 31$ in Fig. 14. Of this difference in N_t, a small portion [$(2.04/2)^2$ or about 4%] results from the value of $t_{.05}$; the major portion results from the fact that we have now specified a different condition, i.e., that the 95% confidence interval (about twice the standard error) on the transformed scale correspond to an interval of $\pm$ 20% of the mean on the original scale.

So far in this section we have considered only the number of trees required to define mean population in one stratum. What is the best allocation of sampling resources when two strata must be sampled in the same stand? Snedecor (32) refers to work of Neyman showing that, when the size and the variance of each stratum is known, the most efficient sampling consists in drawing a number of sample units from each stratum which is jointly porportional to the size and standard deviation of the stratum. In stands that require stratified sampling in the Green River area, it has been shown that the overstory comprises about 75% of total branch surface and the understory 25% (Sec. 9.1), and that, in advanced infestations, standard deviations in the understory may be three or four times higher than those in the overstory (Sec. 11). According to Neyman's rule, therefore, it is correct to sample with about equal intensity in the two strata. In endemic populations or early stages of infestation, variance is about equal in the two strata so it would be correct to reduce sampling intensity in the understory. Hansen *et al.* (14) discuss the same problem and provide formulae for the optimum allocation of samples to different strata, depending on the cost function. Since differences in cost affect the allocation of samples only in proportion to the square root of the relative costs per sample unit, the allocation is scarcely influenced by small cost differences. The authors conclude that little will be gained by considering the cost function unless relative costs are significantly different by a factor of three or more. The cost of sampling the understory for spruce budworm populations is about one-third of the cost of sampling the overstory (Sec. 12.6), and no adjustment for this factor has been made to date.

A final point requiring comment has to do with the number of eggs per mass. In calculating optimum sample size in the present section, and in discussing variance in earlier sections, we have generally referred to egg masses rather than to eggs. However, in using population data in life tables it is necessary to deal with the actual number of eggs rather than the number of masses. If the mean population of masses is defined with a certain precision, will the mean population of eggs be defined with an equal precision? In the course of sampling, it would be very time-consuming to count the number of eggs in each mass found on the sample branches, so we have generally counted only the masses. It is correct, therefore, to deal with masses in determining the mean and variance for a cluster of trees. Each year, however, the number of eggs per mass is determined from a random sample of masses drawn from the total number collected on each plot. Under ordinary conditions, the mean number of eggs per mass averages about 18.5 and the lack of significant variance associated with years or plots permits considerable pooling of data to obtain strong estimates of both $\bar{x}$ and s^2. Under conditions of serious food shortage in advanced infestations, when the size and fecundity of the females are markedly reduced, the mean number of eggs per mass on a plot may be reduced to a low of about 15.7. For different plots that have reached this stage of infestation, pooling of data on eggs per mass is also often permissible. The variance associated with counts of eggs per mass is approximately

$s^2 = 100$. For a given stage of infestation there is generally available, through pooling, measurements of over 1000 egg masses. The standard error of the mean number of eggs per mass is therefore of the order of 0.3. The standard error of the mean number of eggs per 10 sq. ft. is the standard error of a product,

$$s_{(\bar{x}_1\bar{x}_2)} = \sqrt{\bar{x}_1 s_{\bar{x}_2}^2 + \bar{x}_2 s_{\bar{x}_1}^2}$$

where x_1 refers to egg masses per 10 sq. ft. and x_2 refers to eggs per mass. If $\bar{x}_1 = 170$, $s_{\bar{x}_1} = (18.2\%$ of $\bar{x}) = 30.9$ (Fig. 13), $\bar{x}_2 = 18.5$, and $s_{\bar{x}_2} = 0.3$, then $\bar{x}_1\bar{x}_2 = 3145$ and $s_{(\bar{x}_1\bar{x}_2)} = 575$, so $s_{(\bar{x}_1\bar{x}_2)}$ is approximately 18.3% of the mean. That is, the mean number of eggs has been estimated with very nearly the same precision as has the mean number of masses. The sampling error associated with the determination of mean number of eggs per mass may therefore be disregarded, and the precision of mean egg-mass population may be applied to mean egg population. Partial counts of the number of eggs per mass should be continued each year, however, and the results compared to normal values in order to detect any changes of significance.

12.4 Number of Clusters per Plot

The limited data available to date (Table VI) indicate that intercluster variance is not greater than intertree variance within clusters. If this is so, then the mean squares for clusters and for trees are both estimates of the same variance. In Table VI, however, the mean squares for clusters are considerably lower than the mean squares for trees. As the difference is not significant, this must have resulted from a chance selection of clusters that were very similar as to mean population.

TABLE VI

THE VARIANCE BETWEEN CLUSTERS AS COMPARED TO THE VARIANCE BETWEEN TREES WITHIN CLUSTERS

(For each plot, 10 trees were sampled for third-instar larval population in each of two clusters)

Plot No.	Mean larvae per 10 sq. ft.		Mean squares of logarithms (s^2)	
	Cluster 1	Cluster 2	Between clusters	Between trees within clusters
G5	5.67	4.98	.0018	.0628
G2	8.04	8.61	.0220	.0417
Q1	14.8	12.4	.0049	.0570
G4	51.1	62.0	.0167	.0301

Although more extensive data will be secured on intercluster variance, the indicated lack of significance is not surprising. It has been pointed out that plots are particularly located to represent a homogeneous forest condition and a homogeneous stage of infestation. Also, considerable dispersions occur in budworm populations in the moth stage, the first larval instar in the fall, the

second larval instar in the spring, and probably in later larval instars when food is scarce. Within a homogeneous area of 25 acres, therefore, it is unlikely that localized pockets of either high or low population could develop.

It is probably safe to conclude that a sample consisting of a number of trees in one cluster will provide as much information as a sample consisting of the same number of trees drawn entirely at random throughout the whole plot. This is very encouraging; it will simplify design and reduce cost in the sampling of any forest insect to which it is applicable. For it means that the number of clusters per plot is of no importance, once the absence of significant intercluster variance is conclusively demonstrated. The problem is reduced to that of finding the number of trees required to define mean population with the desired precision, as treated in the preceding section, and if it proves more convenient, all the trees may be located in one cluster.

12.5 Influence of Population Level

No doubt it has already been observed that precision and required sample size are influenced by the mean level of population (Table V, Figs. 13, 14). At moderate to high populations, required sample size is fairly constant but at low populations it increases rapidly. This is so whether variance is based on the original or the transformed data (Fig. 14). Although variance is reasonably independent of the mean on the transformed scale, standard errors and required sample numbers are relatively higher at low populations because the transformation has been log $(x + 1)$ rather than log x. Had log x been used, and found appropriate, the standard error would be a constant percentage of the mean. The distribution of the variances for the transformation log $(x + 1)$, however, indicates that the use of log x would not stabilize variance but would result in relatively high variance at the lower levels of population (Figs. 11, 12).

In order to appreciate the practical implications of Fig. 14, it is necessary to know what values of $\bar{x}$ are likely to be encountered at different stages in the epidemiology of the insect. This will be the subject of the following paragraphs.

It is well known that spruce budworm populations become very low in the periods between successive outbreaks in a given area. In fact, the absence of the budworm in general insect survey collections over certain periods of years has led to the supposition that it may even disappear entirely from certain areas. This must remain only a supposition unless it is supported by future work based on collection methods that are more particularly suited to this insect. The actual endemic level of population has not yet been reliably established for the Green River area. Although sampling was initiated in 1945, when the population was very low, there can be no assurance that some increase over the endemic level had not already taken place. Also, the sampling techniques tested during the first three years were more relative than absolute, and it was not until 1948 that the branch surface method was developed and applied. However, through subsequent work on the structure

of the balsam crown, it is possible to transform these early results into reasonably good estimates of population per unit of branch surface. Such transformation indicates that in 1945 and 1946 there were 0.05 larvae, 0.01 pupae, and 0.01 egg masses per 10 sq. ft. To define populations of this order within the limits of precision on which Fig. 14 is based would require over 10,000 trees per plot for larval sampling and over 300,000 for egg-mass sampling, and could only be done if thousands of men were available. This suggests that the development of life tables, even for a single plot, would hardly be feasible at endemic levels of the spruce budworm. This should be accepted only tentatively, as it is based on the extrapolation of relationships shown in Fig. 14 and not on any real knowledge of variance at endemic levels of population. More definite conclusions will be reached by extending the present population work well into the postepidemic period.

A significant increase was first detected in 1947, when mean population was 0.4 larvae and 0.1 pupae or egg masses per 10 sq. ft. (In developing infestations, pupal and egg-mass populations are roughly equal in any one generation.) At this level, mean larval population could be defined with about 280 sample trees but pupal and egg sampling would require about 3000 and 4000, respectively. In 1948, population increased to three larvae and to 0.5 pupae or egg masses, indicating that required sample size would be about 40, 210, and 310 trees, respectively. With a field establishment of some 20 workers, it is probable that satisfactory life tables could be developed for at least one plot at this level of population. At three larvae per 10 sq. ft. the infestation was still extremely light and the defoliation, which amounted to some 2% of the new needles, was scarcely detectable. The next increase gave populations of roughly 20 larvae and five pupae or egg masses, indicating a sample size that would permit the study of several plots (Fig. 14). Further increases in population reduce the required sample size only slightly. The relationship between population and defoliation is influenced by many factors, such as the relative degree of insect mortality before the main feeding period, the intensity of staminate flower production, and the effects of repeated defoliation on the foliage production of the trees. As a general index for new infestations on balsam fir, however, it may be noted that populations of 35 egg masses, 135 larvae, and 35 pupae have corresponded to 100% defoliation of the new shoots during the larval feeding period of the same generation of the insect. About one-half of this population causes about 50% defoliation, and so on. Populations greatly in excess of those required for complete loss of new foliage are frequently encountered (Figs. 9, 10) and this condition may be called 'supersaturation'. It is attended by 'back-feeding' on the unfavorable old needles and by various degrees of starvation. The annual decline in population after this stage is reached, however, appears to be less rapid than the annual increase during the period of development. Plot K1 reached its peak larval population of over 250 in 1951 but by 1954, when trees started to die, this had not decreased below 70.

The possibility that the preparation of reliable life tables will be extremely difficult, if not impractical, at endemic population levels is disappointing. Our knowledge of the epidemiology of any species will be incomplete until we know what factors are responsible for the maintenance of endemic populations over a period of years, and through what mechanism the population succeeds in 'escaping' from the endemic level. It seems probable that the spruce budworm may present unusual difficulties in this respect because of its very low endemic level. Throughout the sampling in the Green River area, the populations of other defoliating insects associated with the budworm have been recorded as incidental data. Although most of these other species have remained endemic, their populations have been several times higher than the budworm population of 1945–46. Assuming that variance is similar to that encountered in budworm sampling, life tables would be feasible for many of them at their endemic levels.

In order to anticipate possible changes in sampling technique for the spruce budworm in the postepidemic period, let us assume for the moment that conclusions reached here through extrapolation prove to be correct and that, with present techniques, no method is found for reducing the standard error in relation to the mean at endemic population levels. Will population work be fruitless ? I believe not, but representativeness and accuracy will have to be sacrificed, to some extent, to permit a reduction in the size of the sample unit and a very great increase in the number of sample units. One-half of a mid-crown branch will probably represent a large enough unit, and such units will be collected in logging areas where large numbers are readily available from newly-felled trees. The use of sampling ladders or platforms for such small units will hardly be economical. Also, if significant intertree variance and correlation cannot be demonstrated at low populations, it may be permissible to draw several units per tree and still treat them as primary sample units. If it is still impractical to continue life tables, the next modification will be to sample only one stage of the insect in order to detect annual changes alone. Even this may provide very useful information on fluctuations and on their predisposing factors. In this event, the larval stage will easily be the first choice. Larval populations are generally about four times higher than pupal or egg-mass populations in the same generation, and consequently the standard errors are much lower in proportion to the means. Also, larvae can be counted very rapidly as soon as their effects on the new shoots are apparent, but before they are large enough to suffer an important degree of dislodgment in the felling of the tree. Larvae collected at this period also provide the necessary data on the most important species of parasites. Modifications such as these would permit many hundreds of sample units to be examined by a relatively small field party, and even if standard errors exceed 100%, any important trends in population will be detected. It has been noted above that increases in larval population starting in 1947 proceeded at the rate of about eightfold per year. Although branch surface was not measured, methods similar to these, involving the sampling of large

numbers of balsam tops in logging operations, were employed in 1945, 1946, and 1947. Statistical significance was demonstrated for the 1947 increase in population, although the total field establishment consisted of only five men and the sampling was divided between two types of stands. The comparison of at least the larval population in different stand types may well be practical, therefore, under low populations. It will be unnecessary to devote any sampling time to understory strata under such conditions (Sec. 9.1).

12.6 The Cost Function

For the sampling design employed in the present work, the cost function, or rather that part of it in which we are interested, may be represented as follows:

$$C = C_1p + C_2pc + C_3pct + C_4pcts,$$

Where, C = total cost,
C_1 = cost of moving from plot to plot,
C_2 = cost of moving from cluster to cluster within plots,
C_3 = cost of moving from tree to tree within clusters,
C_4 = cost of drawing, examining, and checking one sample unit,

And p, c, t, s represent the number of plots, clusters per plot, trees per cluster, and sample units per tree, respectively.

This function may be used in two ways. If the size of the sampling crew cannot be increased, then C is a fixed quantity and the function may be solved to find how many plots, etc., can be studied each season. On the other hand, if C is flexible but there is a required minimum of forest conditions, or plots, which must be studied, then we can estimate the number of men required for the work.

To demonstrate the practical use of the cost function, we shall apply it to tentative plans for the population work in 1955. It is decided to sample two clusters, of 10 trees each, in each stratum. This represents a compromise between the desired precision (Fig. 14, 20 trees per stratum) and the desired variety of stand types and infestation ages. For this type of sampling, $c = 2$, $t = 10$, and $s = 1$. The next step is to determine the cost per plot for the different methods of collecting the samples.

Use of pole pruner from ladder.—A five-man field party is used and the samples can be collected from the two clusters in one day. Plots are at different distances from the laboratory headquarters but the average time for car travel may be taken as 30 min., including the loading and unloading of the ladder. This must be doubled, because C_1 generally consists of a return trip. Therefore $C_1 = 5 \times 1 = 5.00$ man-hours. We have little data on C_2 but the time involved in folding the brace on the ladder, gathering all equipment, and moving 100 yd. or more, may be taken as a minimum of 15 min., so $C_2 = 1.25$ man-hours. The time required to move the ladder from tree to tree within a cluster depends upon how well the trees have been chosen. It takes much longer to lower the ladder and raise it again than it does to swing the ladder

from one tree to an immediate neighbor. If, in any one cluster, it is not necessary to lower the ladder more than three or four times, then the mean time averages very close to 15 min., so that $C_3 = 1.25$. The last cost, C_4, includes collecting time, examining time, and, in the case of egg sampling, checking time. Once the ladder is moved, it takes only eight minutes for the pole operator to select and drop the four branches while the other men measure, clip, and bag them. This represents 0.65 man-hours. The examining time for egg masses is $\frac{1}{3}$ man-hour per 1 sq. ft. of foliage, or 3.67 man-hours per average sample unit of 11 sq. ft. Checking time is $\frac{1}{2}$ man-hour per 1 sq. ft., and if only every second twig in the bag is checked, this makes an average of 2.75 man-hours per sample unit. For egg sampling, therefore, $C_4 = 0.65 + 3.67 + 2.75 = 7.07$. For larval or pupal sampling there is no formal checking, and the examining time is generally lower, except when it is necessary to examine flower clusters for early-instar larvae. In general, C_4 may vary from 1.5 to over 3 man-hours. It is logical, however, to base the calculations mainly on egg sampling; both because of its higher variance and its higher cost per sample unit, egg sampling will generally determine the number of plots that can be studied for life-table purposes. We may now write,

$$C = 5.00p + (1.25 \times 2)p + (1.25 \times 2 \times 10)p + (7.07 \times 2 \times 10 \times 1)p = 174p \text{ man-hours} = 19p \text{ man-days.}$$

Use of pole pruner from ground or trestle.—A four-man field party is used. Travel to plots is reduced to 20 min. because there is no ladder to load, so $C_1 = 2.64$. Travel time between clusters is reduced to 10 min., so $C_2 = 0.68$. Movement between trees within a cluster takes no appreciable time because the pole operator has generally started to collect branches from a second tree while the other men are bagging the last of the foliage from the first tree, so $C_3 = 0$. Collecting the branches requires 0.52 man-hours, because there are now four men instead of five, while examining and checking rates remain unchanged; so $C_4 = 6.94$. Therefore $C = 143p$ man-hours or about $16p$ man-days. As the trestle is constructed before the sampling season, construction time is excluded because it is not a limiting factor.

Sampling of understory.—Collecting time is about the same as that just presented for the use of the pole pruner from the ground. The examining and checking times are reduced to about one-third because understory trees often have sparse, flat foliage. So $C = 50p$ man-hours or about $6p$ man-days.

The estimates used in the above calculations represent average values based on reasonably accurate records of the time required for different sampling operations over a period of several years. It will be appreciated, however, that the treatment has been over-simplified and that the annual cost may vary considerably depending on the experience of the workers, the nature of the plot, the stage of the infestation, and many other factors. The thinning of the branches after repeated defoliation, for example, may considerably reduce the time required for examinations and checks.

There are stands of four major age classes on the Green River Watershed. The 105-year class is generally two-storied and the ladder is used for the upper story, so the cost per plot is 19 + 6 = 25 man-days. The 80-year class can generally be represented satisfactorily by the upper story alone and the cost per plot is 19 man-days. The 55-year class often lends itself to trestle construction and, assuming that stratification according to flower production is not necessary, the cost per plot is 16 man-days. The cost per plot for the 35-year class is also 16 man-days. So the total cost of four plots, one in each age class, is about 76 man-days. We may allot a period of about two weeks in August to egg sampling, remembering that personnel cannot be kept too long from other projects that provide the subsidiary data in life tables. A staff of six or seven men should therefore be able to sample the four plots. The time required for the corresponding larval sampling in June and pupal sampling in July by the same staff will be a little less than one week. This is convenient because the other projects require more time during the larval and pupal stages, more careful timing and a shorter collection period is necessary in these stages (Sec. 4), and weekly sampling on certain intensive plots is also in progress in June and July. Should it be desired to have four plots in the advanced part of the infestation and four in newly-infested areas, the staff must be doubled; and should it be desired as well to replicate the series in mixedwood stands and in sprayed areas, the staff must be quadrupled; and so on. In short, the development of life tables for a variety of forest conditions requires large field establishments. As field establishments are generally limited, great care is necessary in selecting conditions that will not suffer unnatural disturbances, that will represent maximum contrasts, and that will provide the most useful information on the epidemiology of the species.

Depending on the collection method employed, the cost of examining and checking the foliage comprises 74% to 90% of the total cost of egg sampling. This serves to point up earlier remarks concerning the need for improved techniques to reduce the tedious ocular examination (Sec. 5.3). In larval and pupal sampling, the laboratory examination comprises 23% to 28% of the total cost, assuming a mean of 2.0 man-hours for C_4. The presence of staminate flowers may increase this to over 50% in the case of third-instar larvae, and it will also be considerably increased if sampling is conducted in the needle- and bud-mining periods.

The cost function is also very useful in the comparison of different sampling designs. If $C_4 = 2.0$ for pupae, then the sampling of two clusters of 10 trees each requires 72 man-hours, using the cost function and data presented above for the use of the pole pruner from the ladder. For the same number of trees per plot selected by a random rather than a cluster design, $C = C_1 + 20 (C_2 + C_3) + 20C_4$. The value of 5.00 for C_1 must be doubled because the collections cannot be made in one day; the values of C_2, C_3, and C_4 remain the same. Thus $C = 100$ man-hours, as compared with 72 for the cluster design.

13. Absolute Population

All results in preceding sections have been expressed as population per unit of branch surface, which was defined as a basic unit of expression. In the present section will be considered: (1) a method for converting results to absolute population or population per acre; (2) the stability of the basic unit over a period of years. These questions will be treated rather briefly. Data are still being accumulated, as time permits, on foliage quantities per tree and per acre, so a complete mathematical treatment at the present writing would be premature.

Reference has been made to the measurement of total branch surface per tree in groups of felled trees from different stands (Sec. 8.2). Crown measurements were recorded for the same trees, so the regression of branch surface on crown size can be plotted (Fig. 15). By measuring crown width at frequent intervals of crown length, it is possible to derive the true volume of the crown. However, this presents difficulties if it is desired to make comparable measurements on standing trees. For this reason only the greatest crown width has been used and this generally occurs at the base of the crown in balsam fir. By using crown length and greatest crown width the volume of a cone can be computed. This is referred to as 'cone volume' to distinguish it from true

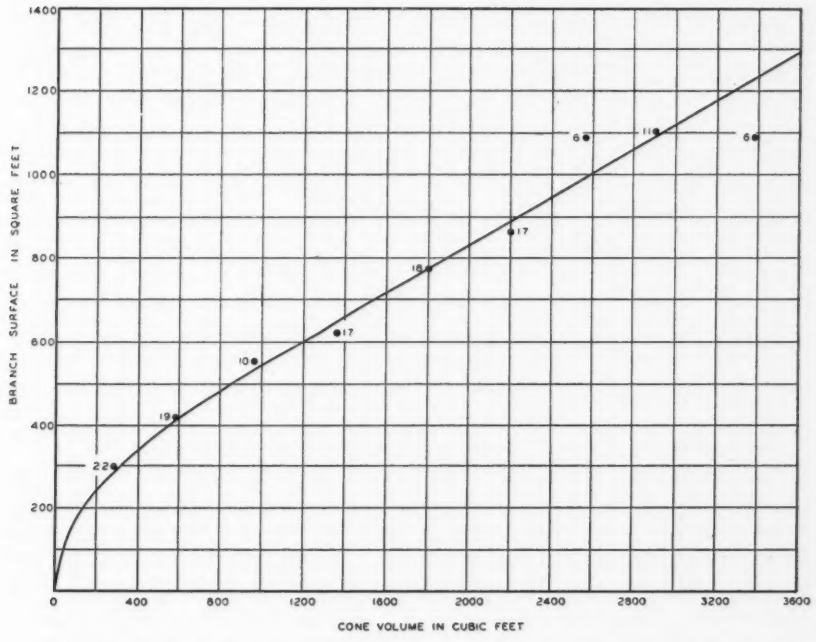


FIG. 15. Regression between total branch surface per tree and volume of the crown, assuming the crown of balsam fir to be represented by a cone of the same height and basal diameter.

crown volume. The form of the crown is not a cone; for 78 balsam crowns varying in length from 15 ft. to 35 ft., crown volume/cone volume = 1.37 ± 0.04. However, cone volume is used here only as an index of crown size and is sufficiently representative for that purpose. The crown measurements apply only to the main body of the living crown; small adventitious branches below the main crown are disregarded.

The next regression needed is that of cone volume on trunk diameter at breast height (D.B.H.). This regression (Fig. 16), which is sometimes used in forestry research, must be based on a large number of measurements because variability is high. With the aid of special instruments, however, the necessary measurements can be made rapidly from the ground on standing trees.

As an index of the scattering of individual points in these two regressions, the standard error of estimate (i.e., standard deviation of the individual trees from the curve) is about 110 sq. ft. for Fig. 15, and varies from 60 cu. ft. for the low D.B.H. groups to 1500 cu. ft. for the high D.B.H. groups in Fig. 16. These preliminary curves have been fitted mainly by freehand methods. It

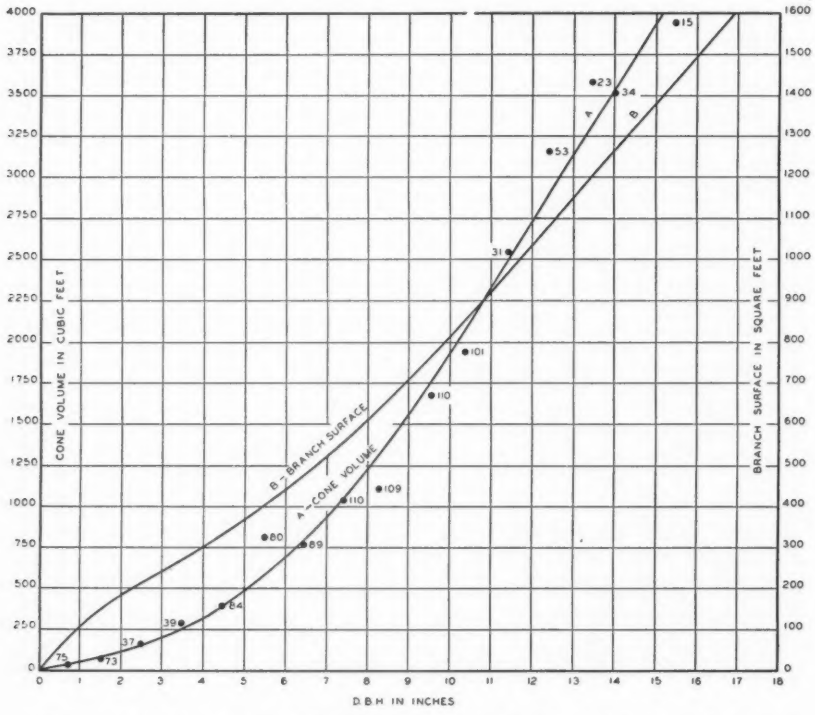


FIG. 16. Line *A*: Regression between cone volume per tree and diameter of the stem at breast height (D.B.H.). Line *B*: Regression between branch surface per tree and D.B.H. The measurements were made on balsam fir on the Green River Watershed.

should also be appreciated that the relationships apply to the Green River area and may be different for other areas, depending on the history and stocking of the stands and the form of the trees. Much, if not all, of the balsam fir in this area is of the variety *phanerolepis* Fern.

From the two regressions the relationship between branch surface and D.B.H. can be plotted (Fig. 16). This final regression can be used in conjunction with ordinary stand tables, showing mean number of trees per acre in each D.B.H. class, to prepare a stand table showing branch surface per acre in each D.B.H. class. The regression is used, therefore, to estimate branch surface not for individual trees but for cruise data based on hundreds of trees. The final step in estimating population per acre is to multiply insect population per unit of branch surface by the total branch surface per acre. The precision with which mean population per acre is estimated can be appraised, but as this involves transformation of the regressions to straight lines it will be deferred until the data are complete.

The change in the quantity of branch surface per acre during the development of a stand can be determined reliably only by successive cruises in the same stand. It will be a number of years before adequate data of this type are available for the Green River stands. Meanwhile, we may obtain a general approximation by comparing branch surface per acre in existing stands of different ages. Such comparison assumes that the existing mature and overmature stands were, at one stage in their development, identical in stocking and growth to existing young stands. Stand history in this area is such (Sec. 9.1) that this assumption seems fairly safe. Mean square feet of branch surface per acre for all plots in different age classes is as follows: 35-year, 270,000; 55-year, 450,000; 80-year, 200,000; 105-year, 120,000. These means apply to the balsam and spruce in softwood stands. Spruce generally represents a minor proportion, and branch surface for a given D.B.H. class is assumed to be the same as for balsam. Work in progress suggests that this assumption is not far wrong. Also, these means include only the overstory. In 80-year stands which have no large openings resulting from budworm or severe blowdown, the understory may contain 15,000 sq. ft.; in 105-year stands it may contain 30,000 sq. ft.; and in stands of either age where normal tree mortality has been hastened by external influences it may contain more foliage than the overstory.

In stands between 35 and 55 years of age, then, branch surface per acre is increasing at the mean rate of 3.4% per year. Therefore, even if population per 10 sq. ft. remains constant in such stands over a period of 10 years, absolute population increases by 34%. Foliage quantity per acre probably reaches its peak near age 55, and then starts to decline. Between 55 and 80 years the mean annual decrement in the overstory is 2.2% per year, and between 80 and 105 years it is 1.6% per year. If we say that mean annual decrement up to age 105 is about 2% per year, then absolute insect population also decreases at this rate if population per unit of branch surface remains constant. The basic unit of population, by itself, is therefore not a reliable index of

absolute population over an extended period of years. For short periods, however, and especially during the violent population changes of an epidemic, the errors involved will not be large until repeated defoliation starts to kill the trees. Then branch surface will decline very rapidly and population per 10 sq. ft. will have little meaning in an absolute sense. This can be rectified either by converting all figures to population per acre or by making the appropriate correction to the estimate of population per unit of branch surface.

Population limits discussed above (Sec. 12.5) may be used in conjunction with the general means for branch surface per acre in order to obtain some appreciation of insect numbers in a given forest area. In the 80-year age class, for example, the very low population of 1945 represents the surprising number of 200,000 $\times$ 0.005 = 1000 larvae per acre, while an epidemic population of 250 larvae per 10 sq. ft. represents 5 million larvae per acre. In dense 55-year stands, the corresponding estimates are 2250 and $11\frac{1}{4}$ million. As numbers of this magnitude are difficult to visualize, it is sometimes helpful to consider the weight, or 'biomass', involved. For example, 90 of the 250 larvae might survive to the pupal stage, and as mean pupal weight is roughly 100 mgm., this would represent nearly 900 lb. of pupae per acre in the 55-year stand. Other applications of population per acre, including its use in connection with vertebrate predator populations, which can be measured only on an area basis, have already been indicated (24). Graham and Shepherd (13) have presented an interesting illustration of its use in forming conclusions as to the possible effect of parasite releases.

Population per tree is also readily estimated. If this is desired for individual trees, the crowns should be measured and the regression of Fig. 15 applied rather than that of Fig. 16. Such estimates have had little application in the present work. They may be useful, however, in preparing life tables for forest insects that pass the larval stage on the foliage and the pupal stage in the ground.

14. Incidental Data

The purpose of this section is to indicate various types of useful incidental data that may be secured, with little additional trouble or cost, during population work. This is something that is worth keeping in mind during the development of sampling techniques, for sometimes slight modifications in technique or in the timing of sampling, will produce very useful data on natural control, fecundity, and other epidemiological factors. The number of man-hours substituted in the cost function includes the recording of associated defoliators, invertebrate predators, and parasite cocoons, but the time taken to record such data represents a negligible fraction of the total cost.

14.1 Population Intensity

Our understanding of the epidemiology of an insect defoliator would be incomplete without some knowledge of its effect on the foliage production and growth of the host tree. Epidemics of some species (and it is quite possible

that the spruce budworm belongs in this category) may owe their decline largely to the intraspecies competition resulting from the exhaustion of the most favorable food sources. Population intensity, expressed as the number of insects per shoot or per unit weight of foliage, is the most suitable expression for use in conjunction with data on annual foliage production, defoliation, and radial growth. A given number of insects per 10 sq. ft. of branch surface may represent widely different population intensities, depending on stand type, flowering habits, and age of infestation.

The same sample branches used for pupal or egg sampling may be used very conveniently to determine the number and the average weight of shoots produced by the tree in the current year, as well as the degree of defoliation resulting from the insect population as determined on the same trees. Foliage production and foliage destruction can thus be expressed in terms of branch surface, and compared directly to population data, and population intensity can readily be expressed. There is the added advantage that the sample unit used in the foliage studies is representative of the whole crown, following the pooling of results for the four levels. Such work logically falls within a separate aspect of the project dealing with defoliation and damage and will be described in more detail by another member of the staff. Meanwhile, if the reader is interested in converting any of the population figures of the present paper to rough estimates of population intensity, the conversion factor of 400 new shoots per 10 sq. ft. of branch surface may be used as a general average for non-flowering trees that have not suffered repeated defoliation.

It may occur to the reader that we might just as well measure the insect population directly in terms of shoot numbers and convert to branch surface afterwards. This would have three disadvantages: (1) the basic and absolute expressions of population would be subject not only to the sampling errors of the insect counts but also to the sampling errors of the shoot counts; (2) the counting of shoots during larval, pupal, and egg sampling would considerably slow down the work during critical sampling periods; (3) some buds remain dormant throughout the season, so in early spring sampling it is not possible to tell which ones will produce shoots. On the other hand, shoot numbers afford great flexibility in the size of the sample unit, and the disadvantages might well be tolerated if the use of shoots showed any promise of reducing intertree variance. Intertree variance does not appear to be significantly reduced, however, when it is based on insects per shoot, and there is no clear evidence of tree-to-tree correlation between insects per 10 sq. ft. and shoot numbers or weight per 10 sq. ft. In other words, the preference shown by the ovipositing moths for certain trees appears to depend on more obscure factors than the number of buds per unit of branch surface.

14.2 Data for Life Tables

The larvae collected in sampling are reared or dissected for parasite and disease organisms; the pupal cases provide data on natural control in the

pupal stage; appropriate measurements on the pupal cases provide estimates of the fecundity of the emerged moths; the empty egg masses provide data on sterility, natural control, and mean number of eggs per mass. The collection of invertebrate predators, parasite cocoons, and associated species of defoliators is also useful. Many of these data could be obtained, of course, by separate collections that are distinct from the actual sampling, and collections of an intermediate nature are often required in any case. However, by using material collected during sampling, we are assured that the criterion of representativeness has been satisfied. It is known, for example, that some parasites may show a preference for certain crown levels, just as the budworm does (15). The nature of the food supply, which affects pupal size and fecundity, also varies with crown level. Thus, random collections of material confined to foliage that is easily reached from the ground are not necessarily representative of the tree or the stand. For this reason the insect specimens obtained from a representative sample unit are particularly valuable. This is why a slight sacrifice in accuracy has been accepted in order to time the sampling so that the maximum of biological data is secured (Sec. 4).

On the same representative sample units, staminate flower clusters are counted each spring during larval sampling. The number of clusters per 10 sq. ft. of branch surface provides an index of flower production in the stand from year to year, and permits the comparison of life tables for budworm populations when staminate flowers differ in their availability to the young larvae.

14.3 Other Sampling Systems

The techniques described in this paper were developed for intensive studies on small plots where the development of life tables is the main objective. However, once the basic work on distribution and variance is completed, the techniques can readily be modified into sampling systems that are applicable for other purposes. This has already been done to meet the needs of extensive surveys (23) and of the biological assay of insecticides (25). Work in progress by another member of the staff permits extension of the technique to other host species so that reliable interhost comparisons are possible based on either absolute population or population intensity.

15. Discussion

Recent philosophical reviews concerning the trend of research in entomology express agreement as to the primary importance of developing adequate techniques for the study of natural populations. For example, Solomon (33) states:

"The prime necessity is a greatly increased flow of basic ecological work. The aim of such work should be to provide an account of the operation of the complex ecological units of which the growing crops are the basis. One useful approach is to study the factors determining the abundance of a particular species of pest, and work out their various interactions. Such work can be

adequate only if it has a quantitative basis, and it requires the best available techniques for the estimation of numbers; no doubt many new or improved methods will be required as this sort of inquiry is developed. Another point is that an adequate ecological study of any pest and its associated species takes a good many man-years of work, and is a job for a team rather than an individual."

In similar vein, Glen (12) writes: "The study of factors that affect insect abundance will probably spearhead the return of ecology as the field of major emphasis in entomology during the next decade..... Once the basic concepts are established and accepted, progress will depend largely on our skill in devising adequate techniques."

It is interesting to note that both of these writers are concerned primarily with agricultural entomology where much of the work, until recent years, consisted of the testing of insecticides. For fruit insects particularly, it has been shown by Pickett and his associates (28) that the application of insecticides without intensive population research may lead to many difficulties.

In forest entomology, where the application of insecticides presents greater problems, the need for adequate population techniques was recognized much earlier by a number of workers. Progress has been slow, however, both because of the obvious difficulties involved in securing samples from tall trees and because adequate field establishments for the necessary teamwork have become available only in recent years.

All this does not mean that the development of sampling techniques is an object in itself; it is, however, the framework around which the ecological program is constructed. This framework must be erected first and, unless it is fundamentally sound, a strong edifice of ecological data cannot be constructed upon it. The development of optimum sampling techniques takes time, and it is an understandable tendency to slight this preliminary phase and get on with the main ecological program. However, until more adequate techniques are available for measuring natural populations and their regulating factors, the study of population dynamics is unlikely to advance beyond the stage of mathematical theory.

The study reported in this paper represents what is perhaps the first attempt to develop a technique for the framework of population data around which life tables are constructed. It is appropriate, therefore, that in this final section we should attempt to say whether or not the whole approach seems to be feasible for forest insects, as based on the progress to date. Can non-sampling errors be held within reasonable limits ?. Are sampling errors low enough to make the estimates worth having ? Probably a general answer cannot be attempted until more pilot work has been done on other species of insects. The study of the spruce budworm on the Green River Watershed presents certain advantageous features: (1) the same foliage sampling unit can be used for all stages; (2) a cluster design appears adequate in homogeneous stands; (3) the stands generally contain only one, or at the most,

two, strata. Disadvantages that may be unusual are: (1) the endemic level of population is apparently lower than it is for other insects; (2) adequate representation in the crown requires a fairly large sample unit, and the collection of branches that are near the apex of tall trees; (3) certain stages are concealed and difficult to sample; (4) the objects of the project require a distribution of effort over as many forest conditions as possible. Very likely, then, some insects will prove easier to handle than the budworm, others more difficult.

It is believed that non-sampling errors can be kept within reasonable limits if special precautions are taken and constant attention is paid to detail. It is necessary, however, that senior men with a very real concern for the accuracy of the population data take an active role in the work. Although it is very tedious, intensive sampling cannot be looked upon as a routine project which can safely be turned over to large crews of casual laborers without adequate supervision. Sampling errors, at moderate or high levels of population, are not prohibitive. It is also probable that they are subject to reduction through further refinements in design and technique as intensive sampling projects become more common. However, in any one season, we must probably be prepared to accept sampling errors that may seem rather large to the laboratory worker. But if the study is initiated with the proper long-term perspective, there will be an opportunity to achieve replication in time, as well as in place, so the formation of erroneous conclusions should be improbable. At endemic levels of population, the required sample size may render it impractical to develop complete life tables for insects that become as scarce as the spruce budworm.

It has been encouraging to learn that a staff of two or three men is sufficient to develop life tables for the budworm in one forest stand (Sec. 12.6). When the study of a variety of stands is an important aspect of the project, much larger field staffs are necessary. The limiting factor in population work is the great amount of time required for the careful ocular examination of the foliage samples. For small stages, such as the egg stage, this part of the work may comprise as high as 90% of the total cost function. Although this represents easy work, it is extremely monotonous. To quote Park (27), "..... population ecology, apart from its exciting theoretical aspects, contains much that is best described as unadulterated drudgery". If this is true of *Tribolium*, which can be separated from its food supply by the use of cloth screens, it is surely an understatement for defoliating insects. Mechanical, electrical, or chemical techniques for separating defoliating insects from the foliage are worthy of exploration and, if their use if feasible, would remove the major limitation from population sampling.

Acknowledgments

It has been indicated in the text that all senior personnel at the Green River Laboratory co-operate with the sampling and its supervision during the major sampling periods. Although the development of sampling design and

technique is the writer's responsibility, the execution of the work is possible only through the wholehearted co-operation of those associates to whom grateful acknowledgment is made in this paragraph. In addition to this type of co-operation, C. A. Miller has undertaken the general supervision and timing of sampling during absences of the writer; D. O. Greenbank has made important contributions to the study of foliage quantities; D. G. Mott, in supervising collecting parties, has made improvements in the operation of the sampling ladder and has done much of the actual climbing; M. M. Neilson and W. F. Cheshire have assisted with the sampling and its supervision; W. F. Cheshire, C. W. Bennett, and R. H. Wightman have assisted with the machine calculations and the preparation of graphs.

I have also been very fortunate in having the fullest co-operation from the Statistical Research and Service Unit, Science Service, Department of Agriculture, Ottawa. Mr. C. Reimer, in particular, has patiently answered my numerous inquiries on statistical problems during the transformation of the data and made many suggestions for improvements in analysis. Dr. R. E. Balch, Officer-in-Charge of the Forest Biology Laboratory, Fredericton, N.B., has given very valuable support to the work, and both he and Mr. Reimer have been kind enough to criticise the manuscript.

Many of the data in the regression of cone volume and stem diameter were kindly made available by the Forestry Branch, Department of Northern Affairs and National Resources, and were accumulated by A. B. Vincent and J. M. Robinson on the Green River Watershed.

References

1. Anscombe, F. J. The statistical analysis of insect counts based on the negative binomial distribution. Biometrics, 5 : 165-173. 1949.
2. Atwood, C. E. The feeding habits of young spruce budworm larvae. Can. Entomologist, 76 : 64-66. 1944.
3. Beall, G. The transformation of data from entomological field experiments so that the analysis of variance becomes applicable. Biometrika, 32 : 243-262. 1942.
4. Blais, J. R. The relationship of the spruce budworm (*Choristoneura fumiferana*, Clem.) to the flowering condition of balsam fir (*Abies balsamea* (L.) Mill.) Can. J. Zool. 30 : 1-29. 1952.
5. Bliss, C. I. and Fisher, R. A. Fitting the negative binomial distribution to biological data and note on the efficient fitting of the negative binomial. Biometrics, 9 : 176-200. 1953.
6. Daviault, L. Application of DDT to control the spruce budworm. Can. Dept. Agr. Div. Forest Biol. Bi-Mon. Progr. Rept. 9(1) : 1-2. 1953.
7. Dowden, P. B. and Carolin, V. M. Natural control factors affecting the spruce budworm in the Adirondacks during 1946-1948. J. Econ. Entomol. 43 : 774-783. 1950.
8. Eisenhart, C. The assumptions underlying the analysis of variance. Biometrics, 3 : 1-21. 1947.
9. Fettes, J. J. Spruce budworm population trends in a section of the Lake Nipigon infestation. Can. Dept. Agr. Div. Forest Biol. Bi-Mon. Progr. Rept. 5(1) : 2. 1949.
10. Finney, D. J. On the distribution of a variate whose logarithm is normally distributed. J. Roy. Stat. Soc. Suppl. 7 : 155-161. 1941.
11. Finney, D. J. An introduction to statistical science in agriculture. John Wiley & Sons, Inc., New York. 1953.
12. Glen, R. Factors that affect insect abundance. J. Econ. Entomol. 47 : 398-405. 1954.

13. GRAHAM, K. and SHEPHERD, R. Population measurement of the lodgepole needle miner and its parasites. Can. Dept. Agr. Div. Forest Biol. Bi-Mon. Progr. Rept. 7(4) : 3-4. 1951.

14. HANSEN, M. H., HURWITZ, W. N., and MADOW, W. G. Sample survey methods and theory. Volume I: Methods and applications. John Wiley & Sons, Inc., New York. 1953.

15. JAYNES, H. A. Parasitization of spruce budworm larvae at different crown heights by *Apanteles* and *Glypta*. J. Econ Entomol. 47 : 355-356. 1954.

16. LEJEUNE, R. R. Private communication. October. 1954.

17. MILLER, C. A. A technique for assessing spruce budworm larval mortality caused by parasites. Can. J. Zool. 33 : 5-17. 1955.

18. MORRIS, R. F. How old is a balsam tree ? Forestry Chron. 24 : 106-110. 1948.

19. MORRIS, R. F. Frass-drop measurement in studies of the European spruce sawfly. Bull. No. 12. School Forestry & Conserv. Univ. Mich. 1949.

20. MORRIS, R. F. Techniques for population sampling on standing trees. Can. Dept. Agr. Div. Forest Biol. Bi-Mon. Progr. Rept. 6(6) : 1-2. 1950.

21. MORRIS, R. F. The effects of flowering on the foliage production and growth of balsam fir. Forestry Chron. 27 : 40-57. 1951.

22. MORRIS, R. F. One source of error in budworm population work. Can. Dept. Agr. Div. Forest Biol. Bi-Mon. Progr. Rept. 7(6) : 1-2. 1951.

23. MORRIS, R. F. A sequential sampling technique for spruce budworm egg surveys. Can. J. Zool. 32 : 302-313. 1954.

24. MORRIS, R. F. and MILLER, C. A. The development of life tables for the spruce budworm. Can. J. Zool. 32 : 283-301. 1954.

25. MORRIS, R. F. and MOTT, D. G. The immediate effects of spraying for the spruce budworm in parts of the Upper Green River and South Branch Kedgwick, 1953. Interim Tech. Rept., Lab. Forest Biol., Can. Dept. Agr. Fredericton, N.B. 1954.

26. MORRIS, R. F. and REEKS, W. A. A larval population technique for the winter moth (*Operophtera brumata* (Linn.) (Lepidoptera: Geometridae)). Can. Entomologist, 86 : 433-438. 1954.

27. PARK, T. Experimental studies of interspecies competition. II. Temperature, humidity and competition in two species of *Tribolium*. Physiol. Zoöl. 27 : 177-238. 1954.

28. PICKETT, A. D., PATTERSON, N. A., STULTZ, H. T., and LORD, F. T. The influence of spray programs on the fauna of apple orchards in Nova Scotia: I. An appraisal of the problem and a method of approach. Sci. Agr. 26 : 590-600. 1946.

29. PREBBLE, M. L. Sampling methods in population studies of the European spruce sawfly, *Gilpinia hercyniae* (Hartig), in eastern Canada. Trans. Roy. Soc. Can. V. 37 : 93-126. 1943.

30. PRENTICE, R. M. The life history and some aspects of the ecology of the large aspen tortrix, *Archips conflictana* (Wlk.). Interim Tech. Rept., Lab. Forest Zool., Can. Dept. Agr. Winnipeg, Man. 1954.

31. REIMER, C. Private communications. 1954.

32. SNEDECOR, G. W. Statistical methods. 4th ed. Iowa State College Press, Ames, Iowa. 1946.

33. SOLOMON, M. E. Insect population balance and chemical control of pests. Chemistry & Industry, pp. 1143-1147. 1953.

34. STARK, R. W. Analysis of a population sampling method for the lodgepole needle miner in Canadian Rocky Mountain Parks. Can. Entomologist, 84 : 316-321. 1952.

35. WELLINGTON, W. G. The light reactions of the spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae). Can. Entomologist, 80 : 56-83. 1948.

36. WELLINGTON, W. G. and HENSON, W. R. Notes on the effects of physical factors on the spruce budworm, *Choristoneura fumiferana* (Clem.). Can. Entomologist, 79 : 168-170. 1947.

OOGENESIS IN SEVERAL FREE-LIVING AND PLANT-PARASITIC NEMATODES[1]

By Roland H. Mulvey[2]

Abstract

Two techniques were used in preparation of nematode material for the present study of nematode chromosomes. These were (*a*) the squash method (using propionic-orcein in staining) and (*b*) the paraffin embedding method. A new technique, using 2% agar blocks, was employed in the handling of small nematodes. Oogenesis in a cyst-forming nematode (*Heterodera* sp.), two root-knot nematodes (*Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949, and *Meloidogyne hapla* Chitwood, 1949), and a free-living nematode (*Diplogaster* sp.), was studied using slide material prepared by the squash method. Chromosome numbers were determined for the most part in the primary oocyte. It is suggested that chromosome numbers may eventually be of some use in the determination of plant-parasitic nematode species.

Introduction

Recent workers have increasingly recognized the economic importance of plant-parasitic nematodes. Life history studies, chemical and cultural control, and taxonomy of plant-parasitic nematodes have received considerable attention. Although the physiology and morphology of the more important nematodes parasitic on plants have been studied, the chromosome numbers and other cytological aspects to date have not been investigated. However, gemetogenesis in animal-parasitic nematodes belonging to the genus *Ascaris* L., 1758, has been studied extensively by many well known cytologists, including Van Beneden (25), Carnoy (3), and Walton (26, 27). The cytology of free-living species of nematodes belonging to the genus *Rhabditis* Dujardin, 1845, has also received considerable attention by Kruger (14), Belar (1), and Honda (11). The present investigations were undertaken after a careful consideration of the above workers' publications.

The purpose of the present paper was to determine the possible diagnostic usefulness of chromosome numbers in nematode taxonomy. Preliminary studies of oogenesis in several plant-parasitic and free-living nematodes form the basis of the present investigation.

Source and Species of Nematodes Studied

Meloidogyne spp.—Root-knot nematodes belonging to the genus *Meloidogyne* Goeldi, 1887, are parasitic on approximately 2000 different kinds of host plants and are therefore easily obtained. Species identification is

[1] *Manuscript received March 18, 1955.*

Contribution from Oregon State College, Corvallis, Ore., from a thesis submitted to Oregon State College, in partial fulfillment of the requirements for a Master of Science degree.

[2] *Research Officer (Agriculture), Nematode Investigations, Ottawa, Canada.*

principally based on the perineal pattern of the female (5). Young gravid females (Fig. 10) selected for study were taken from the following infected host plants:

Meloidogyne hapla, from lettuce roots.

Meloidogyne incognita, from potato tubers.

Meloidogyne incognita, from potato roots.

Meloidogyne incognita, from multiflora rose roots.

Heterodera sp.—A cyst-forming nematode of the genus *Heterodera* Schmidt, 1871, was obtained from hairy vetch roots. An ample supply of gravid females was available 35–37 days after the planting of hairy vetch in pots containing soil heavily infested with *Heterodera* cysts.

Diplogaster sp.—A saprozoic nematode of the genus *Diplogaster* M. Schultze, in Carus, 1857, was obtained from decaying narcissus bulbs. Gravid females of *Diplogaster* sp. were present in sufficient numbers in the bulbs.

Materials and Methods

Two methods of preparing the nematode material were employed: a modified squash method (22) and the paraffin embedding method.

The plant-parasitic female nematodes were fixed in Carnoy type fixative (absolute alcohol 6, chloroform 3, and glacial acetic acid 1) for one to three hours. The nematodes were then transferred to a small watch glass containing propionic-orcein stain (1 gm. of orcein (obtained from the British Drug Houses) dissolved in 100 cc. of 45% propionic acid, which was then brought to a boil, cooled, and filtered). After three to five minutes the nematodes were placed in a small drop of propionic-orcein on an albumenized slide. A cover slip was applied and by gentle pressure on the cover slip the female nematodes were broken open to release the body contents into the stain. The slide was heated several times over an alcohol flame, with care to prevent the fluid from boiling. Excess fluid was removed with filter paper.

Dehydration was accomplished by the vapor exchange method (2). The slides were placed in a Coplin jar, the bottom of which was lined with absorbent paper saturated with 95% ethyl alcohol. The slides were left in the vapor jar for a period of two to four hours and then placed in a 95% ethyl alcohol bath. The cover slip was pried free of the slide and then both cover slip and slide were taken from the alcohol bath and placed on filter paper. Excess alcohol was quickly removed from around the stained nematodes, a drop of diaphane (which is miscible with 95% ethyl alcohol) was placed on the nematode material, and the original cover slip applied. Gentle pressure on the cover slip flattened the nematode eggs to aid in subsequent examination of the chromosomes.

The free-living nematodes were prepared for study in the same manner as that described for the plant-parasitic nematodes except for the time of fixing, which was 15-25 min. Better penetration of the fixative was made possible by the removal of the anterior end of each nematode while in the fixative.

Propionic-orcein stains the chromosomes satisfactorily and at the same time generally imparts very little stain to the cytoplasm of the oocyte. The stained material is best observed after a period of approximately one month. The mounting medium (diaphane) hardens during this time and the nuclear elements show up much better than when the slide was first made. During the staining process the nematodes should be first transferred from the fixative to propionic-orcein stain in a small watch glass. Later the nematodes may be transferred to a small drop of stain on a slide. This additional step prevents "travelling" of the propionic-orcein on the slide caused by repulsion between the fixative and the stain.

The paraffin method of preparing nematodes for staining involves considerable difficulty in handling and, therefore, the following procedure was adopted: A 2% agar solution was prepared and a small watch glass was partly filled with this solution. The living female plant-parasitic nematodes were placed in the center of the warm agar and oriented as desired. After solidification the agar was trimmed into a small rectangular block with the nematodes in the center.

The block was fixed in Allen's modified Bouin's fluid (heat 100 cc. of Bouin's fluid to 37° C. and add 1.5 gm. of chromic acid crystals and 2 gm. of urea) overnight in an oven at 37° C. Several blocks containing nematodes were also fixed overnight in Bouin's fluid.

After fixation the blocks were rinsed in tap water, washed in 50% ethyl alcohol (saturated with lithium carbonate) five times at 20-min. intervals. After it was washed, the material was thoroughly dehydrated in pure dioxan. The dioxan was replaced with xylol which, after two to three minutes, was removed from the vials containing the blocks and was replaced by melted paraffin. The blocks were thoroughly infiltrated with paraffin and then embedded in low-melting-point paraffin. The material was sectioned 10μ in thickness and then mounted on albumenized slides.

The nematode material fixed in Allen's modified Bouin's fluid was stained in crystal violet using iodine (1% aqueous solution) as a mordant after staining. The material fixed in Bouin's fluid was stained in Heidenhain's iron hematoxylin.

Chromosome counts were made using a Wratten (No. 56) green filter during the examination of the nematode material to increase the contrast between the chromosomes and the cytoplasm. Because the chromosomes were at various depths in the spindle careful focusing of the microscope objective was required in determining the number of chromosomes.

Nematode chromosomes in material prepared by the paraffin method did not stain sufficiently for study. The compact chromatin material in the nucleus stains satisfactorily with either crystal violet or iron hematoxylin. The chromosomes may have remained unstained because of poor fixation which might be corrected by using a more penetrative fixative (e.g., Carnoy's fluid).

All drawings were made with the aid of a camera lucida. A 10× eye-piece and a 90× (N.A. 1.25) oil immersion achromatic objective was used in the examination of the chromosomes.

Experimental

A. Oogenesis in Plant-parasitic Genera

Results

(*a*) *Oogonial Development*

The youngest oogonia (Fig. 1, *A*) appear as very small cells in the anterior portion of the nematode ovary. The nuclear diameter is nearly half that of the cell diameter. The chromatin is a single compact rounded mass situated in the center of the nucleus.

The nucleus in mitosis is resolved into individual chromosomes (Fig. 1, *C*, *D*). The spindle fibers are faintly visible. As the oogonia move down the ovary the cells increase in size with corresponding growth of the interphase nucleus. Definite chromosomes are formed after the last of the oogonial divisions which mark the end of the multiplication of the cells. Chromosomes which exhibit a chain-like attachment to each other (Fig. 1, *E*) were observed in the primary oocytes of the *Heterodera* sp. Honda (11) observed a similar condition in *Rhabditis elegans* Maupas, 1900, at the end of the growth period. He reported that the chromosomes appear to be joined end to end.

(*b*) *Oocytes*

The prophase of the first maturation division extends through the period of growth of the sex cells.

As previously mentioned, after the last oogonial division the mass of chromatin in the oogonial nucleus breaks up into chromosomes which appear to be attached end to end. Lin (16) reports that during the strepsinema and diakinesis stages of the meiotic prophase of the *Ascaris* egg the heavily stained chromocenter is resolved into individual heterochromatic ends which later contract; typical *Ascaris* tetrads are then formed. He also observed that the euchromatic parts of the chromosomes stained very lightly and were therefore easily overlooked. Although the meiotic prophase stage was not studied in detail in the present work, structures which appeared to be chromocenters were observed in the *Heterodera* sp. There were approximately 16 of these chromocenters (called prochromosomes by earlier workers) in the primary oocyte (Fig. 1, *E*). Following this stage the chromatin again appears to become concentrated into a mass within the nucleus (Fig. 1, *F*). As the primary oocyte moves down the ovary it increases in size with corresponding growth of the nucleus.

The chromatin is organized into definite chromosomes (Fig. 3, *A*; Fig. 4, *A*) after the completion of the growth period. Duplication of each chromosome follows. The homologous chromosomes pair (Fig. 3, *B*) and thus the primary oocytes have the haploid number of chromosome pairs.

During metaphase the chromosome pairs become arranged at the equatorial plate region (Fig. 1, *G*; Fig. 2, *A*; Fig. 3, *D*; Fig. 4, *B*; Figs. 6–9). The chromosome fibers appear to be in bundles and each bundle of fibers is associated with only one chromosome. The achromatic material between daughter chromosomes is well defined. The cytoplasm is densely vacuolated

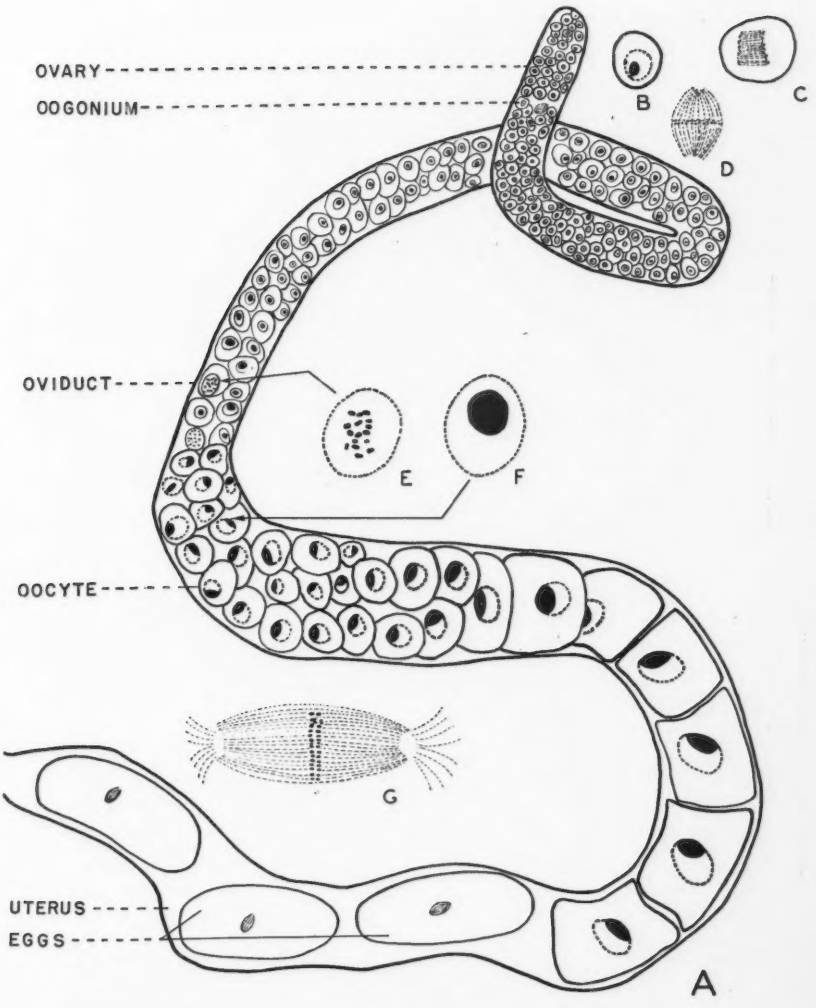


FIG. 1. Female reproductive organ of *Heterodera* sp. *A*, × 450; *B-G*, × 1300.

A, female reproductive organ of *Heterodera* sp. showing multiplication of oogonia and growth of the oocytes; *B*, oogonial cell showing interphase stage of nucleus; *C*, oogonial cell showing metaphase stage of mitosis, side view; *D*, oogonial cell showing late anaphase stage of mitosis, side view; *E*, primary oocytes showing chromocenters of the prophase of the first maturation division; *F*, primary oocytes showing interphase stage of nucleus; *G*, primary oocyte showing chromosomes at equatorial plate region.

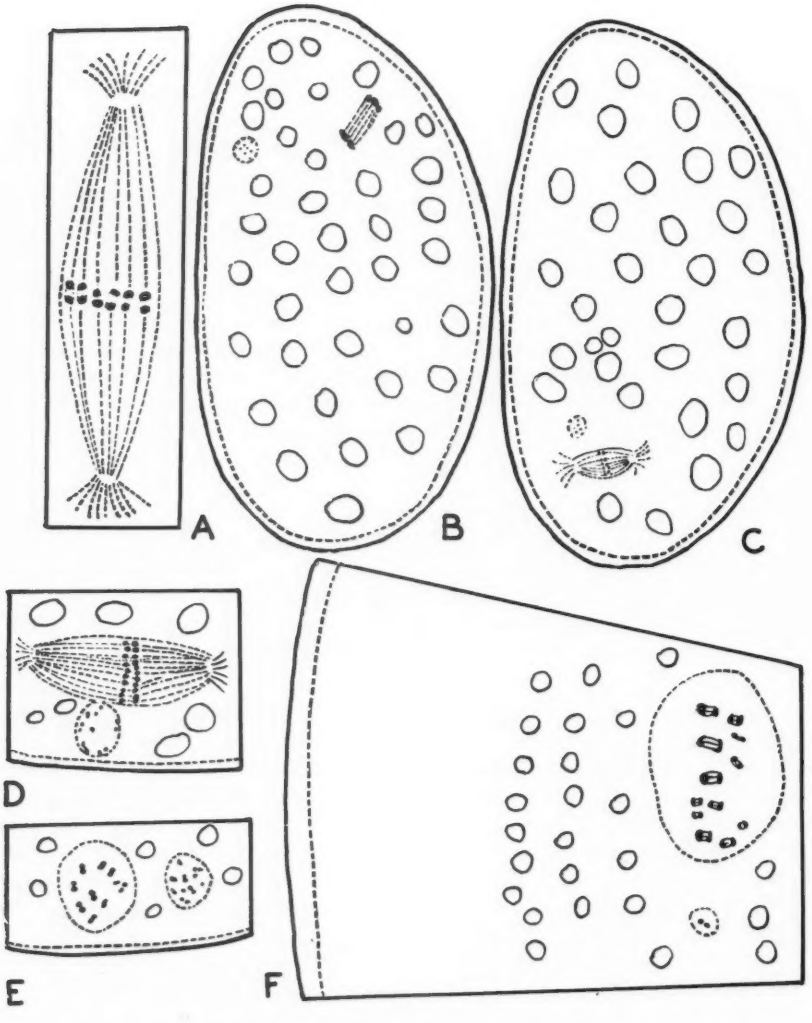


FIG. 2. Maturation stages of *Meloidogyne hapla*. *A*, *D-F*, × 2700; *B*, *C*, × 1900. *A*, primary oocyte showing chromosomes at the equatorial plate region, side view; *B*, primary oocyte showing telophase stage with sperm nucleus within the egg, side view; *C*, secondary oocyte showing early anaphase (note closely associated sperm nucleus), side view; *D*, secondary oocyte showing early anaphase, side view; *E*, male and large female pronuclei, each with haploid number of chromosomes; *F*, "fusion nucleus" and cleavage centrosome.

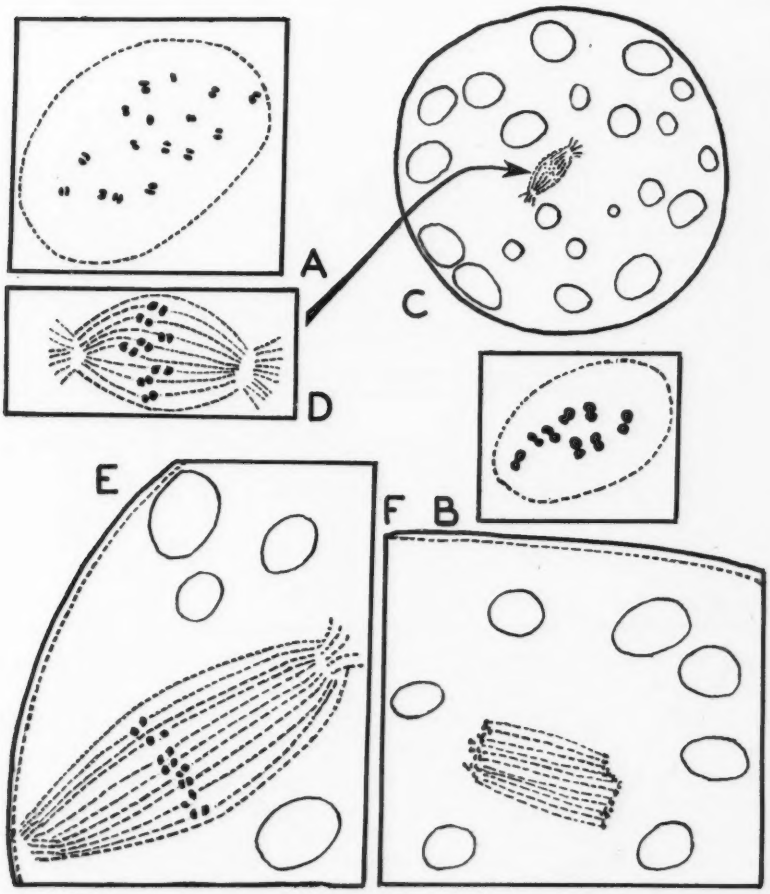


FIG. 3. Maturation stages of *Meloidogyne incognita*, *A*, *B*, *D-F*, × 2700; *C*, × 950.

A, primary oocyte showing prophase nucleus with duplication of each chromosome; *B*, primary oocyte showing prophase nucleus with eight bivalents (observed in the center of an egg); *C*, primary oocyte showing chromosomes at equatorial plate region, egg cytoplasm densely vacuolated; *D*, showing spindle of primary oocyte; *E*, primary oocyte showing chromosomes in equatorial plate region with one pole of the spindle against the fertilization membrane; *F*, oocyte showing telophase stage.

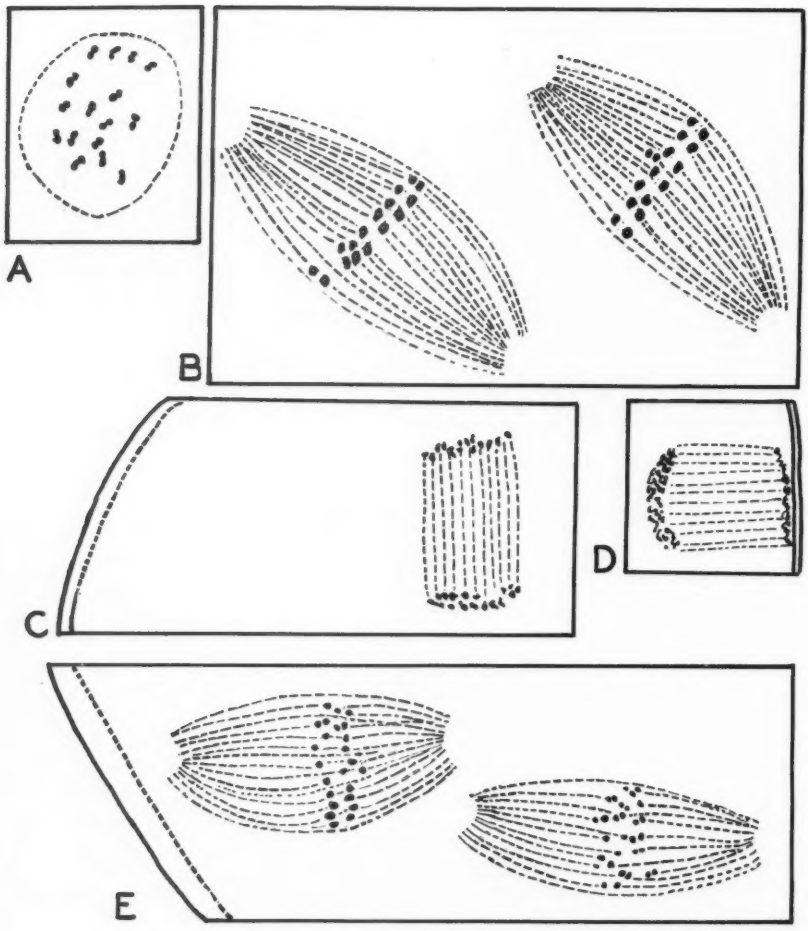


FIG. 4. Maturation stages and cleavage spindle of *Heterodera* sp. × 2700.

A, primary oocyte showing prophase stage; *B*, primary oocyte showing chromosomes in equatorial plate region, chromosomes observed in two planes of same spindle; *C*, primary oocyte showing telophase stage; *D*, primary oocyte showing telophase stage with one pole of the spindle against the fertilization membrane; *E*, cleavage spindles.

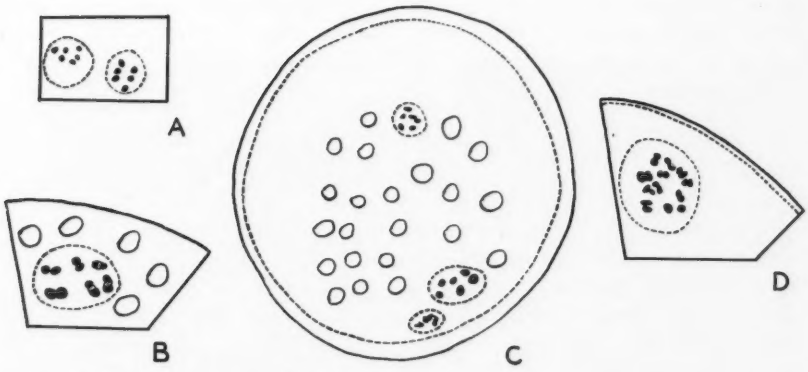


FIG. 5. Maturation stages of *Diplogaster* sp. × 2100.

A, cells found in the female uterus and male seminal vesicle; *B*, primary oocyte showing prophase stage; *C*, secondary oocyte showing prophase stage with secondary oocyte and closely associated polar cell. The sperm nucleus is set off to right of larger oocyte. The egg cytoplasm is vacuolated; *D*, oocyte showing anaphase stage with chromosomes observed at two levels, polar view.

(Fig. 3, *C*) and the membrane is very thin. During early metaphase the spindle and its nuclear elements lie within the center of the egg. Eventually the spindle, which up to this time is parallel with the long axis of the egg, rotates through an angle of 90° and comes to rest with one of its poles against the cell membrane (Fig. 3, *E*).

Many oocytes were observed in early anaphase but few were found in late anaphase and telophase. The two latter stages are probably of short duration. During telophase stage (Fig. 2, *B*; Fig. 3, *F*; Fig. 4, *C*, *D*) the chromosomes become clumped together with the spindle fibers faintly visible between daughter groups. Further development of the daughter groups into the first polar cell and secondary oocyte was not observed.

The prophase stage of the second maturation division was not observed. The second maturation division follows the telophase stage of the first maturation division. The chromosomes become arranged in the equatorial plate region (Fig. 2, *C*). At first the spindle fibers are at right angles to the long axis of the egg. Eventually the spindle undergoes a rotation of 90° and comes to rest with its fibers parallel with the long axis of the egg (Fig. 2, *D*). The chromosomes separate and the telophase stage is initiated. The pronucleus (Fig. 2, *E*) is derived from this last division of the chromosomes in the egg.

(*c*) *Fertilization*

The sperm nucleus was first observed in the plant-parasitic nematodes during telophase of the primary oocyte (Fig. 2, *B*). The sperm is closely associated with the spindle during second maturation division (Fig. 2, *C*). The female pronucleus, after it has become established, comes to lie side by

side with the sperm nucleus (Fig. 2, *E*). Individual chromosomes are clearly visible in both pronuclei. The fusion nucleus (Fig. 2, *F*), which is formed from the fusion of the two pronuclei (male and female), has the diploid number of chromosomes.

B. Oogenesis in a Free-living Nematode, *Diplogaster* sp.

Results

(*a*) *Oogonial and Oocytal Development*

The movement of the oogonia and the oogonial structure (Fig. 11) in *Diplogaster* sp. parallels that previously described for the plant-parasitic nematodes. Chromocenters (prochromosomes), described by Walton (27) and found in the *Heterodera* sp. under discussion, were not observed during the prophase of the first maturation division. According to Walton (27) in *Ascaris canis* Werner, 1782, during prophase of the first maturation division the chromatin mass breaks up into individual chromosomes. Pairing of the chromosomes gives the impression that only the haploid number of chromosomes is present. This latter stage was observed in *Diplogaster* sp. (Fig. 5, *B*).

The maturation stages between prophase and the formation of the first polar body were not studied.

The secondary oocyte shows a peripheral polar cell and a closely associated egg nucleus containing individual chromosomes (Fig. 5, *C*). The sperm nucleus, in which the chromosomes appear discrete, is located to one side of the egg and apart from the egg nucleus. The cytoplasm shows some shrinking away from the fertilization membrane.

Anaphase of the secondary oocyte shows two groups with six chromosomes in each group (Fig. 5, *D*). This is apparently a secondary oocyte because of its proximity to the vulva.

Small cells containing intensely stained spherical chromosomes of uniform size (Fig. 5, *A*) were observed in the uterus region. These cells were numerous in females having several eggs and in many instances were not found in females without eggs. Many cells of a similar type, with chromosomes in various arrangements, were found in the seminal vesicle area of the *Diplogaster* male. Apparently these cells are spermatocytes.

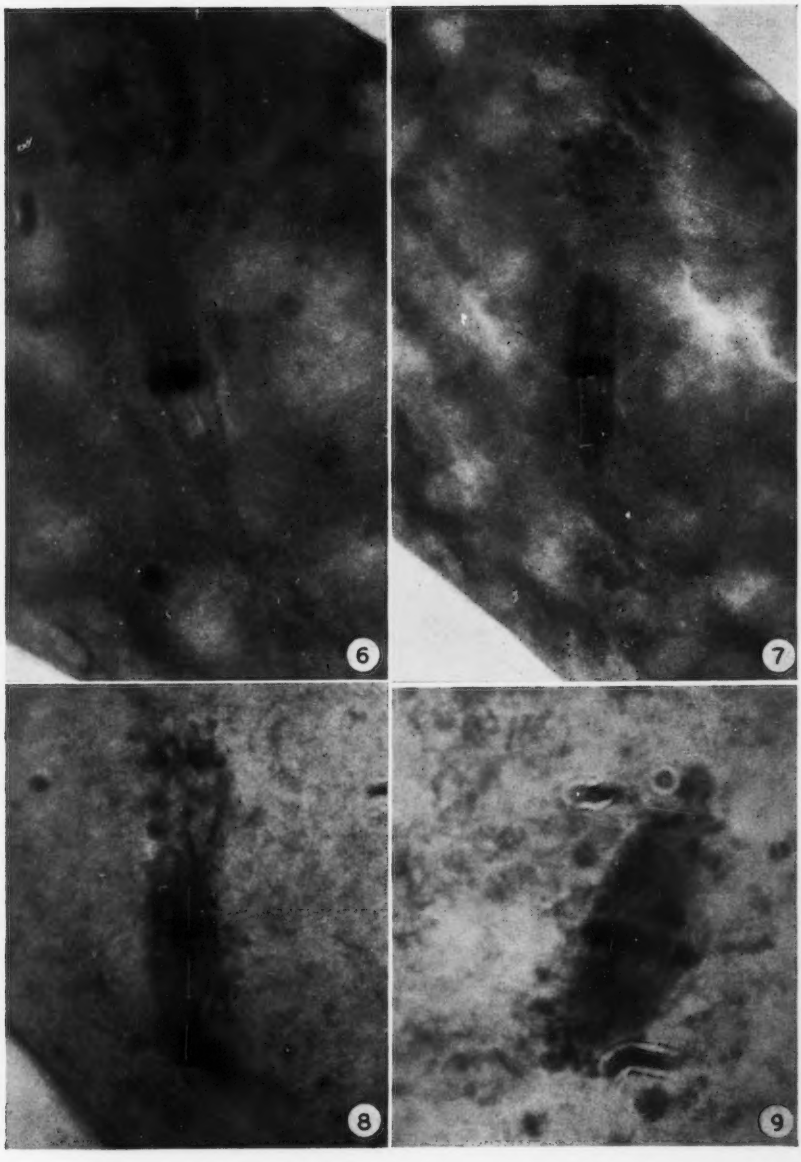
Figs. 6-9, photomicrographs showing primary oocytes of plant-parasitic nematodes. Magnifications: Figs. 6, 7, 9, × 2000; Fig. 8, × 1700.

Fig. 6. *Meloidogyne incognita.* Primary oocyte showing chromosomes in equatorial plate region, level one showing three pairs of chromosomes.

Fig. 7. *Meloidogyne incognita.* Same egg as in Fig. 6, level two showing five pairs of chromosomes.

Fig. 8. *Meloidogyne hapla.* Primary oocyte showing chromosomes in equatorial plate region.

Fig. 9. *Heterodera* sp. Primary oocyte showing chromosomes in equatorial plate region.


6
7
8
9

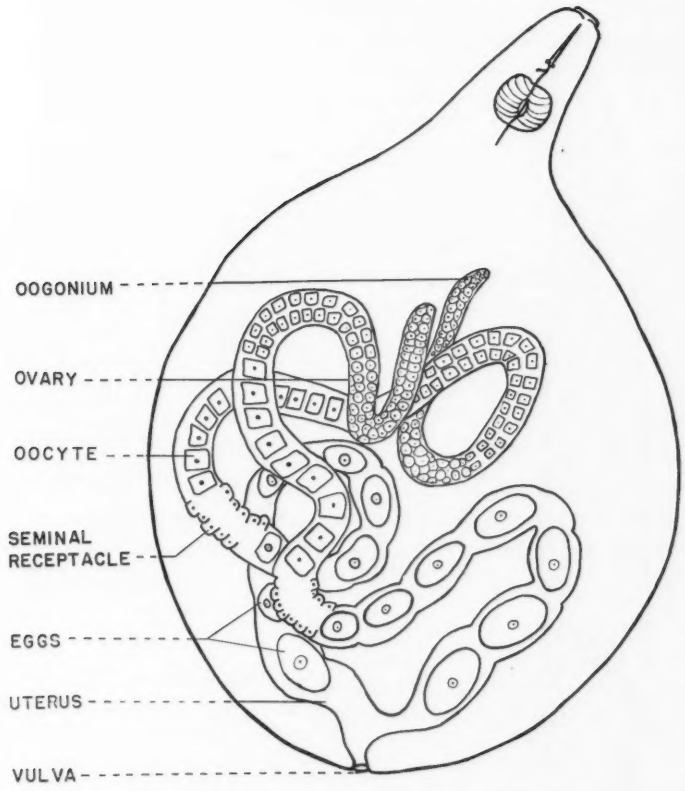


FIG. 10. Reproductive organs of female root-knot nematode, *Meloidogyne* sp. Body outline drawn from a prepared specimen. Reproductive organs are diagrammatic. × 425.

C. CHROMOSOME NUMBERS

Chromosome numbers vary within the *Meloidogyne* species. *Meloidogyne hapla* has a haploid number of 10 chromosomes when the sperm is present and a haploid number of six when the sperm is absent. *Meloidogyne incognita* from potato tuber, potato root, and rose root has a haploid number of eight and a diploid number of 16.

Heterodera sp. from hairy vetch appears to have a chromosome number of 16 (using an achromatic objective) which is probably the diploid number.

Diplogaster sp. has a haploid number of six chromosomes.

All chromosome counts were observed in the primary oocytes. Chromosome numbers of the plant-parasitic and free-living nematodes investigated are incorporated in Table I. Chromosome numbers of free-living *Rhabditis*, according to Makino (17), are also included in Table I.

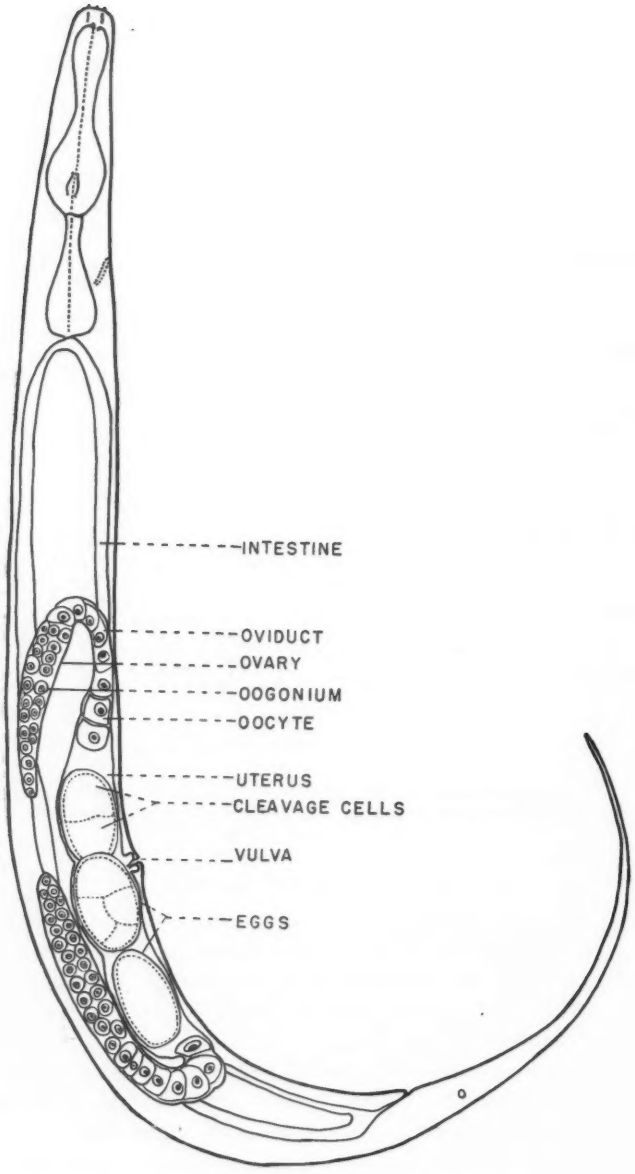


FIG. 11. Reproductive organs of a mature female free-living nematode, *Diplogaster* sp., showing ovaries and cleavage cells. × 450.

TABLE I

CHROMOSOME NUMBERS IN SOME PLANT-PARASITIC AND FREE-LIVING NEMATODES

Species	Source	Chromosome number		Sperm	Males
		2n	*n*		
Meloidogyne hapla	Lettuce	—	6	None	Few
Meloidogyne hapla	Lettuce	—	10	Yes	Few
Meloidogyne incognita	Potato (tuber)	16	8	None	Absent
Meloidogyne incognita	Potato (root)	16	8	None	Absent
Meloidogyne incognita	Rose	16	8	Yes	Few
Heterodera sp.	Hairy vetch	16?	16	None	Absent
Diplogaster sp.	Narcissus	—	6	Yes	Present
*Rhabditis aberrans**	Soil	24	24	Parthenogenetic	
*Rhabditis aspera**	Soil	14	7	Yes	Present
*Rhabditis dolichura**	Soil	—	6	Yes	Present
*Rhabditis elegans**	Soil	—	6	Yes	Present
*Rhabditis gurneyi**	Soil	10	5	Yes	Present
*Rhabditis monohystera**	Soil	—	10	Parthenogenetic	
*Rhabditis nigrovenosa**	Soil	12	6	Yes	Present
*Rhabditis pellio**	Soil	14	7	Yes	Present
Rhabditis pellio (mutant)*	Soil	14	14	Yes	Present
Rhabditis sp.*	Soil	14	7	Yes	Present

NOTE: *Chromosome numbers of Rhabditis spp. were observed in oogonium (2n) and primary oocyte (n).*

* *After Makino (17).*

Discussion

The objective of the present study was essentially to determine the role of chromosome numbers in nematode taxonomy. Recognition of the various maturation and mitotic divisions is, therefore, of great importance. The polar bodies, which are characteristic of certain stages of the maturation divisions, were not observed in the species of plant-parasitic nematodes studied. No explanation is offered at this time for the absence of the polar cells. The interpretations of the various maturation stages are based on structural and physical features of the cell observed during examination of the nematode material. These features are considered further on in the text.

Mitotic divisions in the ovaries of the *Heterodera* sp. show discrete chromosomes (Fig. 1, *C*, *D*), which unfortunately could not be accurately counted. The chromosomes are very small and not entirely separated from each other. Goodrich (9) and Walton (27) observed that the chromatin remains as a single mass during the mitotic divisions of the oogonia in *Ascaris* sp. Although this was not the case in the present studies, mitotic divisions were of little use in the determination of chromosome numbers. The only other mitotic figures observed were those found in a single *Heterodera* egg (Fig. 4, *E*).

The assumption that all figures observed, except those mentioned above, are maturation divisions is based on several factors, including (*a*) the vacuolated cytoplasm of the egg in which the division figures were found, (*b*) the thin cell membrane, (*c*) the duplicated appearance of the chromosomes, (*d*) the presence of sperm nuclei in many cells, and (*e*) the so-called fiber

bundles associated with individual chromosomes. Many eggs in which the cytoplasm had shrunk away from the fertilization membrane showed no stained nuclear material. Several eggs in the two-cell stage of cleavage show the cytoplasm separated from the fertilization membrane. The prophase chromosomes are faintly visible in the two-cell stage.

The limited staining or non-staining of the nuclear material within the eggs described above may be due to the inability of the fixative to penetrate the shell of the more developed egg.

The presence or absence of males should be considered in proposing that chromosome numbers be used as a possible taxonomic character in nematode taxonomy.

Many species belonging to the genera *Meloidogyne*, *Heterodera*, *Rhabditis*, and *Diplogaster* are bisexual, with males and females being present in equal numbers. Males are rare in some species of the forementioned genera and in other species have not been found (Table I).

Therefore, the question arises whether the haploid or diploid number of chromosomes is being observed.

Walton (28) remarks that only in rare instances does the egg develop without fertilization by the sperm. He mentions several workers who have found exceptions or modifications to the above condition. Belar (1) reported that two parthenogenetic species of *Rhabditis* show a single maturation division and no reduction in the chromosome number. A similar condition was found in *Heterodera* sp. studied in the present investigations. No males were found in the mass of host material examined. Sperm nuclei are absent during the maturation division of the eggs. Furthermore, two spindles (Fig. 4, *E*), which may represent a cleavage stage, were found in one egg. Apparently no polar bodies are formed and the telophase of the primary oocyte is the extent of the maturation division. It was not definitely determined whether the diploid or haploid number of chromosomes is present in the maturation division of *Heterodera* sp. The chromocenters found in the ovary are approximately 16 in number within each cell. The first maturation division figures also show at least 16 chromosomes in each daughter group. Considering the two spindles found in one egg as cleavage spindles (each had a chromosome number of 16 or more), apparently the diploid number has not been reduced in the maturation stage of *Heterodera* sp.

Kruger (14) reports that the hermaphrodite *Rhabditis aberrans* Kruger, 1913, produces eggs that are apparently parthenogenetic of the diploid type (one polar body and no reduction of the somatic number of 18). The sperm frequently enters the egg but fails to take part in the subsequent nucleus cleavage. Paula Hertwig (cited by Walton (28)), investigating a dioecious culture of *Rhabditis pellio* (Schneider, 1866) Butschli, 1873, found a mutant which produced only one polar cell without reduction and, therefore, retained the diploid number of 14 chromosomes. None of the eggs would develop without the sperm being present in the egg although the sperm did not enter the cleavage nucleus. In the present investigation sperm nuclei were observed in many of the eggs showing maturation divisions in *Meloidogyne hapla* taken

from lettuce roots. The eggs in which a sperm nucleus is present show a chromosome number of 10 (*n*). Eggs in which a sperm nucleus is absent have a chromosome number of 6 (*n*?). The difference in chromosome numbers within this group may indicate that actually two species are present. A single species with some individuals being parthenogenetic and others bisexual is unlikely since the haploid chromosome number is different in individual females.

Tyler (24) reported that reproduction without males in a root-knot nematode (*Meloidogyne* spp.) is regular and normal. Males, when present, are in many instances rare. She also observed that males appear more frequently in old, unhealthy, or heavily parasitized roots. Males of *Meloidogyne* species, when found during the present study are also extremely few in number as compared with the female population. The oocyte generally contains no sperm nucleus when no males are observed (Table I).

The maturation divisions in the oocytes are best for determining chromosome numbers in plant-parasitic nematodes. The chromosomes in the oocytes are largest and are most readily stained at this stage with propionic-orcein.

The fixative apparently is unable to penetrate the egg shell formed during late maturation and early cleavage. Therefore, under these conditions mitotic figures are not demonstrated.

Early anaphase of the first maturation division appears to be the optimum time for chromosome counting. The chromosomes are positioned slightly apart and early anaphase figures predominate in numbers over all other stages. Considerable pressure (using the eraser of a lead pencil) should be applied to the cover slip of the prepared slide several days after mounting to bring the chromosomes to a common plane.

The present investigation indicates that chromosome numbers may be of some value in the taxonomy of plant-parasitic and free-living nematodes. Apparently the chromosome numbers in the primary oocytes may be used to separate the genera *Heterodera* and *Meloidogyne*. However, several species of each genus should be examined before the role of chromosome numbers as a specific separation can be determined.

Acknowledgments

The writer wishes to express his appreciation and gratitude to Dr. Harold J. Jensen under whose supervision this work was accomplished. Helpful advice was also received from Dr. E. J. Dornfeld, Dr. F. H. Smith, and Dr. H. K. Phinney. The author is grateful to fellow graduate students of the Departments of Botany and Plant Pathology and Zoology for the many ways in which they have assisted the progress of this research. The study was conducted at the Oregon State College, Corvallis, Oregon, using the facilities of the Department of Botany and Plant Pathology. The author is indebted to the Dominion Government of Canada for some financial assistance provided during the course of his postgraduate work at Oregon State College.

References

1. BELAR, K. J. Über den Chromosomenzyklus von parthenogenetischen Erdnematoden. Biol. Zentr. 43 : 513-518. 1923.
2. BRIDGES, C. B. The vapor method of changing reagents and of dehydration. Stain Technol. 12 : 51-52. 1937.
3. CARNOY, J. B. La vésicule germinative et les globules polaires de l'*Ascaris megalocephala*. La Cellule, 2 : 1-111. 1886.
4. CARNOY, J. B. La segmentation de l'œuf chez les nématodes. La Cellule, 3 : 61-113. 1887.
5. CHITWOOD, B. G. A revision of the genus *Meloidogyne* Goeldi, 1887. Proc. Helminthol. Soc. Wash., D.C. 16 : 90-104. 1949.
6. COBB, N. A. Nemic spermatogenesis. J. Heredity, 16 : 357-359. 1925.
7. COBB, N. A. Nemic spermatogenesis with suggested discussion of simple organisms. J. Wash. Acad. Sci. 18 : 37-50. 1928.
8. DOUGHERTY, E. The role of free-living nematodes in genetic research. Proc. 8th Intern. Congr. Genet. Stockholm. Hereditas, 562-564. 1948.
9. GOODRICH, H. B. The maturation division in *Ascaris incurva*. Biol. Bull. 27(3) : 147-150. 1914.
10. GOODRICH, H. B. The germ cells in *Ascaris incurva*. J. Exptl. Zool. 21(1) : 61-99. 1916.
11. HONDA, H. Experimental and cytological studies on bisexual and hermaphrodite free-living nematodes with special reference to the problem of sex. J. Morphol. and Physiol. 40 : 191-233. 1925.
12. JOHANSEN, D. A. Plant microtechnique. McGraw-Hill Book Company, Inc., New York. 1940.
13. KRÖNING, F. Studien zur Chromatinreifung der Keimzellen. Arch. Zellforsch. 17 : 63-85. 1923.
14. KRÜGER, E. Fortpflanzung und Keimzellenbildung von *Rhabditis aberrans* nov. sp. Z. wiss. Zoöl. 105 : 87-124. 1913.
15. LACOUR, L. Acetic-orcein: A new stain-fixative for chromosomes. Stain Technol. 16 : 169-174. 1941.
16. LIN, T. P. The chromosomal cycle in *Parascaris equorum* (*Ascaris megalocephala*): Oogenesis and diminution. Chromosoma, 6 : 175-198. 1954.
17. MAKINO, S. Chromosome numbers in animals. 2nd ed. Iowa State College Press, Ames, Iowa. 1951.
18. MAUPAS, E. Mode et formes de reproduction chez les nématodes. Arch. zool. exptl. et gén. VIII (3) : 463-624. 1900.
19. PASTEELS, J. Recherches sur le cycle germinal chez l'*Ascaris* (Étude cytochimique des acides nucléiques dans l'oogénèse, la spermatogénèse et le développement chez *Parascaris equorum* Goerze). Arch. biol. 59 : 405-446. 1948.
20. SCHLEIP, W. Das Verhalten des Chromatins bei *Angiostomum* (*Rhabdonema*) *nigro-venosum*. Arch. Zellforsch. 7 : 85-138. 1911.
21. SHARP, L. W. Introduction to cytology. 3rd ed. McGraw-Hill Book Company, Inc., New York. 1934.
22. SMITH, S. G. Techniques for the study of insect chromosomes. Can. Entomologist, 75 : 21-34. 1943.
23. THRELKELD, W. L. and HENDERSON, M. E. Spermatogenesis and oogenesis in *Haemonchus contortus* a nematode worm parasite in the fourth stomach of ruminant animals. The Virginia Acad. Sci. 172. 1941. *Abstracted in* Virginia J. Sci. 2(6) : 1941.
24. TYLER, J. Reproduction without males in aseptic root cultures of root-knot nematode. Hilgardia, 7 : 373-388. 1933.
25. VAN BENEDEN, E. Recherches sur la maturation de l'œuf et la fécondation *Ascaris megalocephala*. Arch. biol. 4 : 265-640. 1883.
26. WALTON, A. C. Studies on nematode gametogenesis. Z. Zellen- u. Gewebelehre, 6(2) : 167-239. 1924.
27. WALTON, A. C. Oogenesis and early embryology of *Ascaris canis* Werner. J. Morphol. 30 : 527-603. 1918.
28. WALTON, A. C. Gametogenesis. *In* B. G. Chitwood's An introduction to nematology. Sect. II, Pt. I. M. B. Chitwood, Babylon, N.Y. 1940. pp. 205-215.
29. WILSON, E. B. The cell in development and heredity. 3rd ed. The MacMillan Co., New York. 1925.

INVESTIGATIONS OF THE MODE OF ACTION OF STRAINS OF *BACILLUS CEREUS* FR. AND FR. PATHOGENIC FOR THE LARCH SAWFLY, *PRISTIPHORA ERICHSONII* (HTG.)[1]

By A. M. Heimpel[2]

Abstract

There is a significant correlation between the pathogenicity for the larch sawfly *Pristiphora erichsonii* (Htg.) of *Bacillus cereus* Fr. and Fr. strains and their respective abilities to produce lecithinase. Species of the genus *Bacillus* that are incapable of producing lecithinase are not pathogenic for the larch sawfly. Histopathological evidence of toxemia during the progress of *B. cereus* infection of the sawfly larvae is presented and it is noted that the tissue degeneration is not of the type usually associated with proteolytic or lipolytic activity. Finally, approximately three micrograms of a lecithinase preparation (containing approximately 21 mouse LD_{50} per milligram) was found to be the LD_{50} for the fifth-instar larva of the larch sawfly. The main conclusion drawn from this evidence is that lecithinase plays an important role in the invasion and destruction of infected larch sawfly larvae.

Introduction

Surveys for disease organisms effective against the larch sawfly were made in northwestern Ontario during the years 1949 to 1952. The incidence of disease among the feeding larvae is normally low (12). Most of the bacteria isolated from dead larvae were strains of *Bacillus cereus* Fr. and Fr. A few strains of bacteria from the families *Micrococcaceae* and *Bacteriaceae* were found in dead larvae but feeding tests showed them to be non-pathogenic for the larch sawfly.

The isolation of *B. cereus* strains pathogenic for the larch sawfly (13) was of considerable interest, because according to Steinhaus (24), strains of *B. cereus* had been considered as pathogens only of some Lepidoptera. Several strains of *B. cereus*, including Lepidoptera pathogens, are now known to kill the larch sawfly, and in fact it appears probable that other sawfly species may also be susceptible to *B. cereus* infection.

The purpose of this investigation was to establish the mode of action of *B. cereus* during the process of invasion and destruction of the sawfly larvae. It was intended that the results be used, if possible, to develop a rapid test to 'screen' the virulent strains of *B. cereus* from among the bacterial isolates obtained from the field.

Since most bacterial pathogens of vertebrates cause damage to or death of the host by enzymatic means (streptococcal and clostridial hyaluronidases, staphylocagulases, and clostridial phospholipases) or by biochemical reactions to bacterial poisons (botulinus, tetanus, and diptherial toxins), it was postulated, as a basis for this investigation, that the mode of action in the insect was also of the former type.

[1] *Manuscript received May 11, 1955.*
Contribution No. 221, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.
[2] *Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada.*

There were three reasons for the choice of lecithinase C as the most probable agent of virulence. First, lecithinase is a lethal toxin for vertebrates. Second, *B. cereus* (and variants) and related species (*B. thuringiensis*, *B. sotto*, *B. cereus* var. *alesti*, and some strains of *B. anthracis*) are the only species in the genus that produce phospholipases (8, 17). Third, high lecithinase-producing strains of *B. cereus* readily kill the larch sawfly larvae, whereas other non-lecithinase producing species of the genus *Bacillus* tested, including *B. subtilis*, *B. megaterium*, *B. para-alvei*, *B. pumilis*, and *B. galleriae*, proved non-virulent.

Chu (7) and Bond and Colmer (5) published comprehensive studies of the phospholipase produced by *B. cereus* and most of the pertinent information regarding the kinetics of the enzyme can be obtained from these papers.

The phospholipase produced by *B. cereus* attacks both lecithin and cephalin and might therefore be considered an enzyme complex. Herein the correct and general term, phospholipase, will be applied broadly to the enzyme produced by *B. cereus*, whereas the action of the bacterial products upon the insect will be restricted to observations made upon the lecithinase portion of the complex.

Very little work has been done with phospholipids in invertebrates. However, these substances have been shown to exist in insects (4, 9, 10, 27), and there is reason to believe that the phospholipids in invertebrates and vertebrates have similar functions (22).

Quantitative Lecithinase Tests

Materials

Both a glycerinated preparation and the powder form of the enzyme, phospholipase C, were prepared. The glycerinated preparations were found to be relatively stable, remaining unchanged for more than a year at this laboratory. Chu (7) reported that this type of preparation showed no change in activity in two years. The powdered enzyme appeared to denature very readily when in solution at room temperature. It was accordingly stored at $-10°$ C. over P_2O_5.

Preparation of Glycerinated Enzyme

Five lots of glycerinated enzyme were prepared. The first three lots (I, II, and III) were made from *B. cereus* strain Pr-1017 culture filtrates of nutrient broth. However, the proteins, other than the phospholipase, contained in the culture filtrates, were precipitated with the toxic material. To overcome this difficulty lots IV and V were prepared from cultures grown in a medium lacking proteins. The formula of the medium was:

Potassium phosphate	1 gm.
Magnesium phosphate	0.2 gm.
Sodium ammonium phosphate	1.5 gm.
Proteose peptone	5 gm.
Dextrose	2 gm.
Distilled water	1 liter

This medium proved to be adequate for growth of the bacteria and for good production of the enzyme. Except for this difference in the medium, the five lots of enzyme were prepared in the same fashion.

Bacillus cereus strain (Pr-1017) was grown (for 20 hr. at 34° C.) in nutrient broth (Difco) made up in distilled water (or in the protein-free medium). Twenty grams of kieselguhr per liter were added to 10-liter lots of bacterial culture and allowed to settle. The supernatant was drawn off and filtered through No. 9 Mandler filter cones. The crude enzyme was precipitated from the clear filtrate by two-thirds saturation with ammonium sulphate. The precipitate formed a firm scum on the surface; this scum was easily harvested and immediately redissolved in 1/20th of the original volume of distilled water. This concentrated solution of the enzyme was dialyzed in cellophane against running tap water for 24 hr. To the salt-free solution, one-eighth of its volume of a saturated ammonium sulphate solution was added. The resultant fine, dark-brown precipitate was removed and the supernatant dialyzed against running tap water for 10 hr., then against 50% aqueous glycerol for 24 hr. at 2° C., and then stored at 0° C.

All lots of glycerinated enzyme gave a positive test for lecithinase activity.

Preparation of Dry Powder Enzyme

A sixth lot of enzyme (lot VI) was a preparation of enzyme powder made from culture filtrates of bacteria grown in the special medium mentioned above.

Sixty liters of *B. cereus* (Pr-1017) culture were centrifuged in a Sharples continuous-flow centrifuge, the clear supernatant being treated with a two-thirds saturation of ammonium sulphate. A scum formed and was removed and redissolved in two liters of distilled water. One liter of a saturated solution of ammonium sulphate was added and after the dark precipitate had settled the supernatant was dialyzed in cellophane against running tap water for three days.

Ammonium sulphate to the amount of 1.45 gm. per ml. was added to the dialyzed solution and the resultant precipitate was removed and redissolved in 150 ml. The enzyme solution was dialyzed against running tap water for 24 hr.

The lecithinase was precipitated again from solution by the addition of 0.42 gm. per ml. of ammonium sulphate. The scum was collected and redissolved in 100 ml. of distilled water. Dialysis of the solution against running tap water was carried out for 18 hr.

The 240 ml. of enzyme solution remaining after dialysis were reprecipitated by 1.92 ml. of a saturated solution of ammonium sulphate. Two precipitates were formed; one that floated and gave a strong lecithinase reaction and one that settled out of solution and gave a very weak lecithinase reaction. Both precipitates were retained and were redissolved separately in 100 ml. amounts of distilled water. These solutions were dialyzed against running tap water for 18 hr. Both precipitates were recovered by freeze drying under vacuum over P_2O_5 and stored at $-10°$ C.

Tests of the supernatants recovered at each step in this process showed that there was a loss of lecithinase at each stage. Since the prime purpose of this work was to prepare a stock of lecithinase in as pure a form as possible, no further attention was paid to the heavy precipitate other than to substantiate the lecithinase tests by means of mouse injections of the material. The substance was not toxic to mice by intravenous injection (see section on mouse injections).

Dry Powder Preparation from Bacillus megaterium Cultures

A control preparation was made from a non-lecithinase-producing species of the genus *Bacillus*. *B. megaterium* was selected, since it does not produce lecithinase and yet is apparently close to *B. cereus* taxonomically (6). A parallel run using 60 liters of *B. megaterium* in the same medium was carried out at the same time as lot VI was made. It was noted that approximately one-third as much precipitate was obtained from the *B. megaterium* culture filtrate as from the *B. cereus* culture. All the supernatants and the final substance obtained from the run with *B. megaterium* were negative with respect to lecithinase activity.

Preparation of Egg-yolk Saline

A suspension of lecithovitellin from fresh egg yolks was made by beating washed egg yolks in isotonic saline (two yolks to 400 ml. of 0.9% NaCl). The yolk granules were allowed to settle and the supernatant was siphoned off. If concentration was desired the suspension was kept at 0° C. for two days, and after some of the water had frozen, the rest of the condensed lecithovitellin was siphoned off. Otherwise the suspension was sterilized by Seitz filtration and stored for periods of three or four days at 0° C.

Method

The strains of *B. cereus* and other *Bacillus* spp. tested were grown in nutrient broth (Difco) for 20 hr. Six milliliters of each broth culture were pipetted into sterile spectrophotometer cuvettes. To each cuvette was added 1 ml. of borate buffer solution (pH 7.1). Each cuvette was placed in the spectrophotometer and a reading of the turbidity was made immediately after 0.5 ml. of egg-yolk saline had been added. The lecithin-culture mixture was then incubated at 34° C. for 30 min. and a second spectrophotometer reading was made; any change in turbidity was recorded.

Since the egg-yolk saline preparation was never the same for any two series of lecithinase tests, a standard was required. With each group of bacterial cultures tested, at least three tubes of standard glycerinated enzyme preparation were set up. One milliliter of the enzyme was diluted in 5 ml. of sterile nutrient broth in each cuvette, and 1 ml. of borate buffer (pH 7.1) was added. One-half milliliter of egg-yolk saline was added just before the reading was taken and the same procedure was followed as with the cultures.

To standardize the results so that one series might be compared with another, the standard enzyme replicate readings for each series were averaged and the resultant mean enzyme reading was divided into each of the readings of the individual test cultures. Since action of the standard enzyme preparation upon the lecithin emulsion is in proportion to the concentration of lecithinase, the standard enzyme readings used as a common denominator tended to cancel the variation between series due to inconstant concentrations of the lecithin suspensions.

Tests of the bacterial cultures were first made using the culture without removal of the bacteria. The first three lots of enzyme preparations were used for these experiments.

The cell-free supernatants of centrifuged bacterial cultures were tested for their lecithinase content. Eight of the previously tested strains were grown in nutrient broth for 20 hr. The bacterial cultures were then centrifuged at 10,000 r.p.m. for one-half hour and the supernatants were tested. As a control, a second tube containing bacterial culture was run through the test with each tube of cell-free culture broth.

Results and Discussion

Lecithinase Tests of Bacterial Cultures

The results of the tests of bacterial cultures are recorded in Table I. In these experiments, three separate lots of standard enzyme were used and a significant difference was expected between the readings made. No attempt was made to equalize the titer of the three enzyme lots.

An analysis of variance was carried out on the data and the findings are recorded in Table II.

The analysis of the data shows highly significant differences between the readings from the different enzyme lots. However, with any one standard enzyme preparation, the variation in the rate of production of lecithinase, between the individual bacterial strains, is highly significant.

Apparently four separate readings of lecithinase production by a strain of bacteria are adequate, providing one lot of standard enzyme is used for all readings.

Since the error was low, the standard enzyme preparation must have remained unchanged and did not deteriorate noticeably in storage under the specified conditions.

Lecithinase Tests of Cell-free Supernatants of Bacterial Cultures

The results of these experiments are given in Table III. An analysis of these data was carried out and the findings are given in Table IV. The difference between the strains is highly significant and there is also a significant difference between the tubes containing the bacteria and the corresponding bacteria-free tubes. This latter difference is a depressant effect and is likely due to the presence of the bacteria; however, it apparently has no effect upon the interstrain ratio of lecithinase production.

TABLE I

RESULTS OF QUANTITATIVE LECITHINASE TESTS OF BACTERIAL CULTURES

Bacterial strain	Readings*							Mean
	1	2	3	4	5	6	7	
Enzyme Lot I								
Pr-1011	0.71	0.78	0.82	0.67	—	—	—	0.75
Pr-1017	2.29	2.16	2.16	2.65	—	—	—	2.32
Ma-1040	2.37	1.98	2.09	2.67	—	—	—	2.28
Mu-3013	2.48	2.29	2.11	2.87	—	—	—	2.44
Mu-3055	2.69	2.29	2.62	3.09	—	—	—	2.68
TA-17	0	—	0	0	—	—	—	0
NRS-996	2.22	2.10	1.79	2.44	—	—	—	2.14
NRS-1124	0	—	0	0	—	—	—	0
Enzyme Lot II								
Pr-1011	0.38	0.25	0.37	0.31	0.29	—	—	0.32
Pr-1017	1.58	1.41	1.52	1.60	1.54	—	—	1.53
Ma-1040	—	1.13	1.22	1.43	1.18	—	—	1.24
Ma-2534	—	—	0.92	1.08	1.78	—	—	1.29
Mu-3013	—	1.36	1.44	1.67	1.83	—	—	1.58
Mu-3055	1.76	1.45	1.68	1.87	—	—	—	1.69
TA-17	0	0	0	0	0	—	—	0
NRS-996	1.50	—	1.41	1.43	—	—	—	1.09
NRS-1124	0	0	0	0	—	—	—	0
Enzyme Lot III								
Pr-1011	1.14	0.21	0.83	0.77	0.87	0.85	0.67	0.76
Pr-1017	1.83	2.38	1.74	1.66	—	1.56	2.08	1.88
Ma-1040	1.79	1.93	2.14	2.13	1.73	1.89	1.93	1.93
Ma-2534	1.63	—	1.24	1.17	2.20	1.08	1.29	1.43
Mu-3013	1.46	1.25	1.96	1.90	2.31	1.89	1.98	1.82
Mu-3055	1.51	1.08	2.11	2.12	2.20	1.92	2.11	1.86
TA-6	—	—	0	0	0	0	0	0
TA-7	—	—	0	0	0	0	0	0
TA-9	0.68	0.87	0.88	0.63	0.68	—	—	0.75
TA-10	—	—	1.47	1.60	1.64	1.12	1.53	1.47
TA-17	—	—	0	0	0	0	0	0
TA-18	—	—	0	0	—	0	0	0
TA-20	—	—	0	0	—	0	0	0
TA-21	—	—	0	0	0	0	0	0
NRS-996	1.57	1.98	1.72	1.81	1.64	—	—	1.74
NRS-1124	—	—	0	—	0	—	0	0

NOTE: *TA-6, B. laterosporus; TA-7, B. para-alvei; TA-9, B. galleriae; TA-10, B. cereus (Bacillus "C" Sokoloff); TA-17, B. subtilis; TA-18, B. megaterium; TA-20, B. cereus var. mycoides; TA-21, B. pumilis; NRS-996, B. thuringiensis; NRS-1124, B. megaterium.*

** Each reading represents a ratio of the* $\frac{\text{Strain lecithinase turbidity measurement}}{\text{Standard enzyme turbidity measurement}}$.

TABLE II

AN ANALYSIS OF VARIANCE OF RESULTS OF THE QUANTITATIVE LECITHINASE TESTS OF BACTERIAL CULTURES

Source of variants	Sum of squares	D.F.	Variances	*F* ratio	*P* value
Between bacterial strains	19.9627	6	3.3271	70.6391	$<.01$
Between experiments	9.2576	2	4.6288	98.2760	$<.01$
Interaction	1.1437	12	0.0953	2.0234	$>.05$
Between groups of readings	30.3640	20	1.5182		
Within groups of readings (error)	2.9662	63	0.0471		
Total	33.3302	83			

TABLE III

RESULTS OF QUANTITATIVE LECITHINASE TESTS OF CELL-FREE, CENTRIFUGED SUPERNATANTS OF BACTERIAL CULTURES

Bacterial strains		Readings* 1	2	3	4	5	6	Mean
Enzyme Lot III								
Pr-1011	w.b.	0.90	0.96	0.86	—	0.57	0.57	0.77
	w.o.b.	0.96	0.99	0.96	0.63	0.94	0.59	0.85
Pr-1017	w.b.	1.84	1.89	2.02	1.46	1.46	1.98	1.78
	w.o.b.	2.20	2.06	2.20	2.44	2.02	2.17	2.18
Ma-1041	w.b.	1.16	1.19	1.22	—	1.28	1.37	1.24
	w.o.b.	1.20	1.30	1.38	1.93	1.10	1.20	1.35
Ma-2534	w.b.	0.98	0.99	0.98	—	0.93	1.07	0.99
	w.o.b.	1.04	0.03	1.05	—	1.02	1.20	0.87
Mu-3013	w.b.	1.22	1.17	1.23	—	1.14	1.53	1.26
	w.o.b.	1.38	1.32	1.40	2.08	1.34	1.61	1.52
Mu-3055	w.b.	1.91	2.04	2.08	—	1.52	1.82	1.87
	w.o.b.	2.23	2.11	2.34	—	2.03	2.06	2.15
TA-10	w.b.	1.26	1.60	1.96	—	1.26	1.28	1.47
	w.o.b.	2.00	1.75	2.09	1.92	1.86	1.42	1.84
NRS-996	w.b.	1.37	1.56	2.13	—	1.13	1.53	1.84
	w.o.b.	2.11	1.79	2.29	2.24	2.00	1.71	2.02

NOTE: *w.b. Bacterial culture.*
w.o.b. Cell-free, supernatant of centrifuged bacterial culture.

* *Each reading represents a ratio of the* $\frac{\text{Strain lecithinase turbidity measurement}}{\text{Standard enzyme lecithinase turbidity measurement}}$.

TABLE IV

AN ANALYSIS OF VARIANCE OF RESULTS OF QUANTITATIVE LECITHINASE TESTS OF CELL-FREE SUPERNATANTS AND BACTERIAL CULTURES

Source of variation	Sum of squares	D.F.	Variances	*F* ratio	*P* value
Between bacterial strains	13.4966	7	1.9280	81.6949	$<.01$
Between tubes (with and without bacteria)	0.9860	1	0.9860	41.7797	$<.01$
Interaction	0.3200	7	0.4571	1.9369	$>.05$
Between groups of readings	14.8026	15	0.9868		
Within groups of readings (error)	1.5086	64	0.0236		
Total	16.3112	79			

The process of removing the bacteria by centrifugation was time consuming and apparently of small value in indicating a relative difference in lecithinase production between the strains.

These results show that various strains of *B. cereus* produce lecithinase at different rates and are fairly consistent in this behavior. Confirmation of the statements (5, 7) that members of the '*B. cereus* group' are the only species of the genus *Bacillus* capable of producing lecithinase was obtained for the species tested here.

Pathogenicity Tests

Previously the consensus was that *B. cereus* was specifically a pathogen of Lepidoptera (18, 19, 24). Since 1950, strains of *B. cereus* have been reported as pathogens of three species of Lepidoptera: *Ephestia kuehniella* Z. (16), *Colias philodice eurytheme* Boisduval (25), and *Carpocapsa pomonella* (L.) (26, 20). Earlier reports of the pathogenicity of *B. cereus* are summarized by Steinhaus (24).

Several strains of *B. cereus* were isolated from dying and dead larch sawfly larvae in 1950. Some of these strains were fed to healthy larvae set up on larch foliage in lantern-globe rearing chambers. The results of these preliminary feeding tests suggested that the strain Pr-1017 warranted further study. Laboratory and field feeding tests were repeated in 1951 and the results have been published (13). Further laboratory tests with this strain, and with several other species of the genus *Bacillus*, were conducted in 1952 and 1953 as reported herein.

Materials and Methods

Spore material was obtained by growing the organism on cellophane laid over a semisolid medium according to the method of Reed and McKercher (21). The spores were collected and washed before drying. Dry spore material was stored in sterile vials until required. Spore suspensions were made by mixing spore powder with sterile distilled water.

In carrying out a test, larch twigs were dipped in spore suspensions, dried, placed in lantern-globe rearing chambers, and healthy larvae were placed on them to feed. Control groups were set up on foliage dipped in sterile water. Larvae that died were usually examined within 24 hr.

Results and Discussion

In 1951, mortality as high as 60% and 39% was obtained in laboratory and field feeding tests respectively (13).

In 1952, the laboratory feeding tests were set up in much the same fashion as in 1951, with 15 to 20 fourth- and fifth-instar larvae in each globe. Several additional species of bacteria were tested. The results of these tests are given in Table V.

TABLE V

MORTALITY CAUSED BY VARIOUS SPECIES OF BACTERIA FED TO THE LARCH SAWFLY: 1952

Bacterial strain	Number of tests	Number of larvae	Total number dead	Mortality due to bacterium	
				Number	Percentage
B. cereus (Mu-3055)	3	56	22	21	38
B. cereus (Pr-1017)	5	108	39	38	36
B. thuringiensis (NRS-996)	4	65	25	22	36
B. cereus (Mu-3013)	2	35	14	10	32
B. cereus (TA-10)	2	40	11	8	22
B. cereus (Ma-1041)	2	35	13	6	21
B. cereus (Ma-2534)	3	55	14	9	18
B. cereus (Pr-1011)	2	36	8	3	10
B. megaterium (NRS-1124)	2	40	6	0	0
B. laterosporus	2	40	4	0	0
B. para-alvei	2	40	11	0	0
B. subtilis	2	40	4	0	0
B. cereus var. *mycoides*	2	40	9	0	0
B. megaterium	2	40	4	0	0
B. pumilis	2	40	3	0	0
Controls		190	6	0	0

In 1953 the feeding tests were repeated, but most of the species that consistently gave negative results in 1952 (Table VI) were omitted. All these tests were replicated at least three times using 50 fourth- and fifth-instar larvae per globe (i.e. per test).

TABLE VI

MORTALITY CAUSED BY VARIOUS SPECIES OF BACTERIA FED TO THE LARCH SAWFLY: 1953

Bacterial strain	Number of tests	Number of larvae	Total number dead	Mortality due to bacterium	
				Number	Percentage
B. cereus (Mu-3055)	4	200	91	81	40.5
B. cereus (Pr-1017)	4	200	102	62	31.0
B. thuringiensis (NRS-996)	4	200	71	42	21.0
B. cereus (Mu-3013)	5	250	61	41	16.4
B. cereus (TA-10)	4	200	52	33	16.5
B. cereus (Ma-1041)	4	200	65	21	10.5
B. cereus (Ma-2534)	4	200	45	25	12.5
B. cereus (Pr-1011)	4	200	36	7	3.5
B. megaterium	3	150	6	0	0
Controls		450	16	0	0

These experiments indicate that strains of *B. cereus* and *B. thuringiensis* are pathogenic for the larch sawfly albeit the degree of virulence for the insect is variable between specific strains. The other species of bacteria tested are not virulent for the larch sawfly. This implies that the agent responsible for the virulence of *B. cereus* and *B. thuringiensis* is not produced by other members of the genus *Bacillus*.

Histological Investigations

Strains of *B. cereus* that proved relatively virulent when fed to healthy larvae caused certain unexpected symptoms in the infected larvae. Instead of becoming flaccid and changing color after death, infected larvae retained a normal appearance, except for a slight yellowing of the ventral body in some cases. Otherwise, the progress of the disease was what would usually be expected. Within 10 to 18 hr. of ingesting *B. cereus* spores, infected larvae ceased feeding and diarrhoea and vomiting were often observed.

When bacteria were injected into the body cavity, marked changes in color took place within 8 to 10 hr. and the body wall became shrunken and flaccid. This suggests that death of larvae infected with *B. cereus* is not usually preceded by a bacterial septicaemia.

Series of insects were examined histologically after they had fed on contaminated foliage and the slides were compared with others made from normal feeding insects.

Materials and Methods

Larvae were reared in lantern globes as described previously. The larvae were sampled at regular intervals and, following examination, were classified according to their physical state and their response to stimuli.

1. Sluggish

These larvae had ceased feeding, were wandering, and were slow in responding to such stimuli as gentle pinching with forceps or tapping on the head capsule with a dull needle.

2. Comatose

Larvae in this group responded lethargically to a strong stimulus such as a heated needle brought close to the larva.

3. Moribund

For this class two criteria were established: (1) larvae that failed to respond to the strongest stimuli (actual touching of the larvae with a heated needle); (2) those that did not bleed when a proleg was cut. There is an intentional avoidance here of the term "at point of death", because tissues die at different rates and an attempt to set the time of death would be merely to name one stage in a continuous and rather lengthy process. Some of the larvae classed as moribund were kept at room temperature for set intervals of time before being fixed and prepared for histological examination; others were fixed and prepared immediately.

Sampled larvae were fixed in Smith's modification of Kahle's fluid, dehydrated by use of an *n*-butyl alcohol series (23), and stained with basic fuchsin, with picric acid as a counter stain.

Results

Examination of the slides made from larvae classed as "sluggish" and "comatose" shows that damage done to most tissues in the body precedes the appearance of bacteria in the haemocoele.

Fig. 1 shows an abdominal nerve ganglion of a comatose larva. A characteristic loosening of the neurilemma, vacuolation of the axon cells, and some signs of nuclear degeneration clearly differentiate it from the normal ganglion depicted in Fig. 2. Bacteria are absent from the haemocoele but are visible in the lumen of the Malpighian tubule at the upper right of Fig. 1.

Fig. 3 shows part of an infected larval salivary gland. In this insect infection was via the sericos to the salivary gland, as well as per os. Bacteria are visible in the lumen of the badly degenerated gland, but again there are no bacteria in the haemocoele. The normal salivary gland from a healthy insect is shown in Fig. 4.

When larvae are infected with *B. cereus*, multiplication of the bacteria in the gut causes changes in the midgut cells. The peritrophic membrane, present in healthy larvae (Fig. 6), disappears (Fig. 5). Pycnosis of the nuclei is evident, and basophilic granules accumulate in the distal end of the cells (Fig. 7). Even when the changes are evident, there is no evidence of proteolysis and the bacteria are not necessarily present in the blood.

Muscle tissue also may be noticeably altered before the bacteria penetrate to the blood, as shown in Fig. 8. This was a "comatose" larva, and there is loss of distinct striation and separation of fibers as compared with muscle from a healthy larva (Fig. 9).

Discussion

The evidence presented shows that the body tissues of the larch sawfly larvae, during the process of infection by *B. cereus*, begin to degenerate (regardless of the location of the bacteria) in the following sequence:

First the midgut cells are damaged; this is accompanied by the complete dissolution of the peritrophic membrane. The salivary gland tissue, the Malpighian tubules, and finally the muscles, nerve tissues, and hypodermal cells begin to degenerate. Bacteria may enter the blood through a rupture in the midgut wall at any time after the gut is seriously damaged. Usually the gut is ruptured at either or both ends of the midgut, where there are sections of delicate cells joining the midgut to the crop and hind intestine. The insect may cease to respond to stimuli and to all appearances is moribund before bacteria reach the blood. As pointed out by Bird (3), there are other phenomena that cause similar symptoms in the midgut cells (e.g. changes in the midgut cells of moulting sawfly larvae), consequently the damage done to the insect tissue in the haemocoele despite the absence of bacteria from the blood is perhaps more significant.

Since larch sawfly larvae infected with *B. cereus* show no evidence of paralysis prior to death, as occurs during the normal course of infection of *Bombyx mori* L. with *Bacillus sotto* Ishiwata (2), the mode of action of the two disease organisms appears to be distinct.

It is postulated that *B. cereus* produces a soluble toxin that kills the larvae, regardless of whether the bacteria enter the blood before death.

Larch Sawfly Injections

Materials and Methods

Six groups of fourth- and fifth-instar larvae of the larch sawfly were injected with various concentrations of sterile, purified phospholipase solutions made from Lot VI enzyme preparation. The enzyme powder was weighed and dissolved in distilled water and this solution was sterilized by Seitz filtration. A control group of fourth- and fifth-instar sawfly larvae were injected with a preparation made from *B. megaterium* broth culture. A vacuum holder, previously described (14), was used to hold the larvae while the injections were made and the largest quantity injected, per insect, was 1 microliter.

Several groups of fourth- and fifth-instar larvae were subjected to the introduction of small amounts of sterile enzyme into the midgut. Although the insects died in a much shorter time (18 hr.) than is taken during the typical case of the disease (30 to 36 hr.) the presence of bacteria in the blood was detected in all cases of midgut injection, preventing any conclusions regarding the effectiveness of the enzyme in killing the insect. Unless one resorts to rearing sterile insects for midgut injections, it is not possible to test the effect of the purified enzyme in the gut, since the larvae will not feed on foliage that has been dipped in the enzyme concentrate.

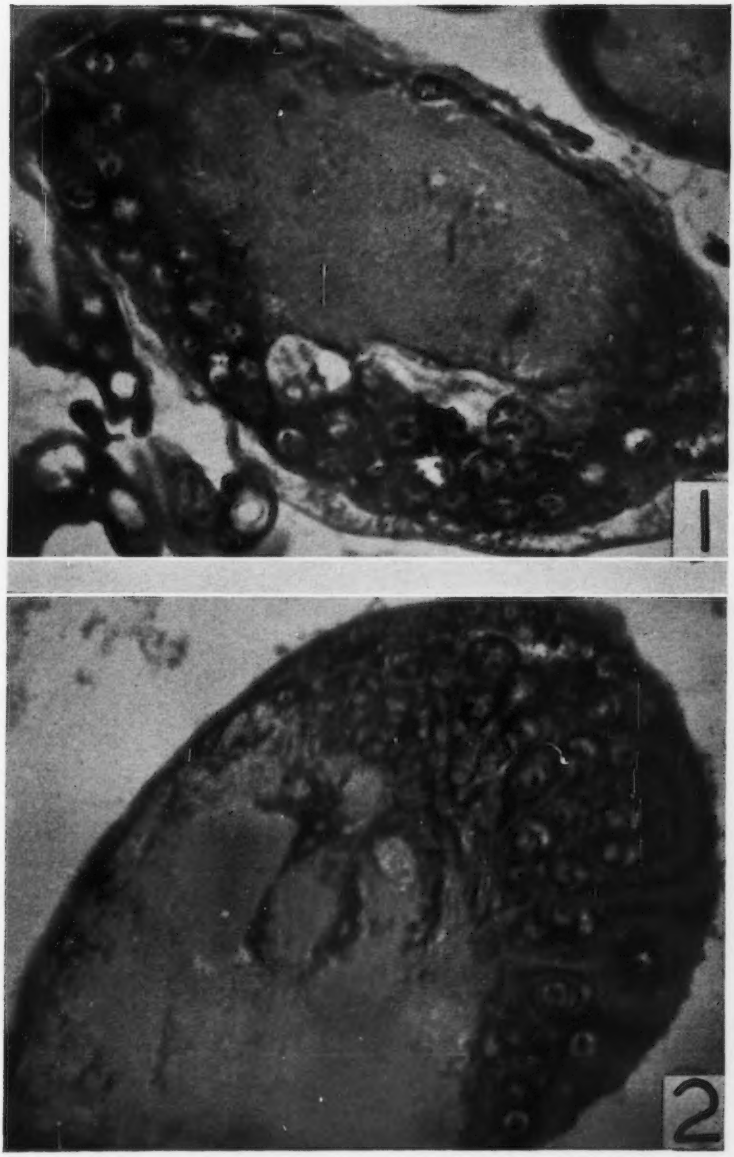
Results and Discussion

The results of the enzyme injections are recorded in Table VII. The LD_{50} for the sawfly is approximately 0.003 mgm. of the preparation made at this laboratory; this dosage corresponds to 0.062 of an LD_{50} for the mouse (see section on mouse injections). There were no deaths in the controls throughout the experimental period, indicating that the deaths in the test group were probably not due to trauma.

TABLE VII

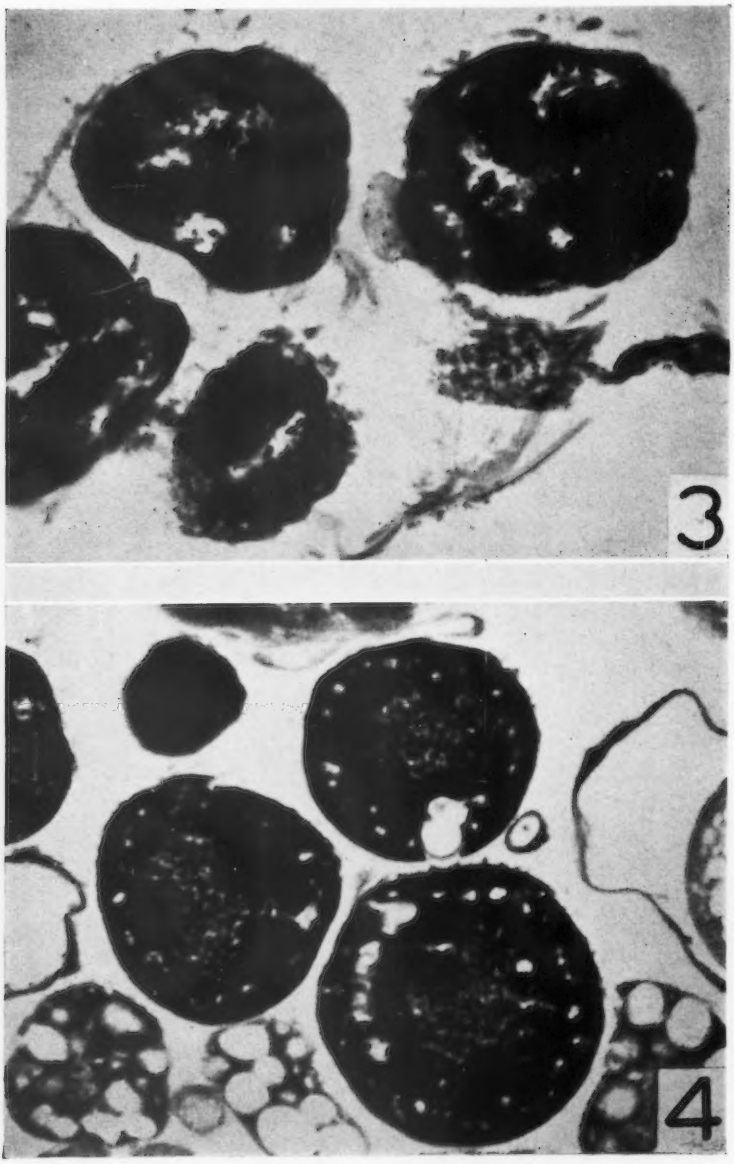
Results of injection of $(NH_4)_2SO_4$ precipitates from *B. cereus* and *B. megaterium* into the haemocoele of larch sawfly larvae

Test	$(NH_4)_2SO_4$ precipitate from	Dosage per larva	Number of larvae injected	Number larvae dead in 24 hr.
1A	*B. cereus* (lot VI)	0.002 mgm.	25	3
1B	*B. cereus* (lot VI)	0.004 mgm.	25	18
1C	*B. megaterium*	0.004 mgm.	25	0
2A	*B. cereus* (lot VI)	0.002 mgm.	25	0
2B	*B. cereus* (lot VI)	0.004 mgm.	25	20
2C	*B. megaterium*	0.004 mgm.	25	0
3A	*B. cereus* (lot VI)	0.003 mgm.	25	11
3B	*B. megaterium*	0.004 mgm.	25	0
4A	*B. cereus* (lot VI)	0.003 mgm.	25	13
4B	*B. megaterium*	0.004 mgm.	25	0
5A	*B. cereus* (lot VI)	0.003 mgm.	25	11
5B	*B. megaterium*	0.004 mgm.	25	0
6A	*B. cereus* (lot IV)	0.003 mgm.	25	13
6B	*B. megaterium*	0.004 mgm.	25	0

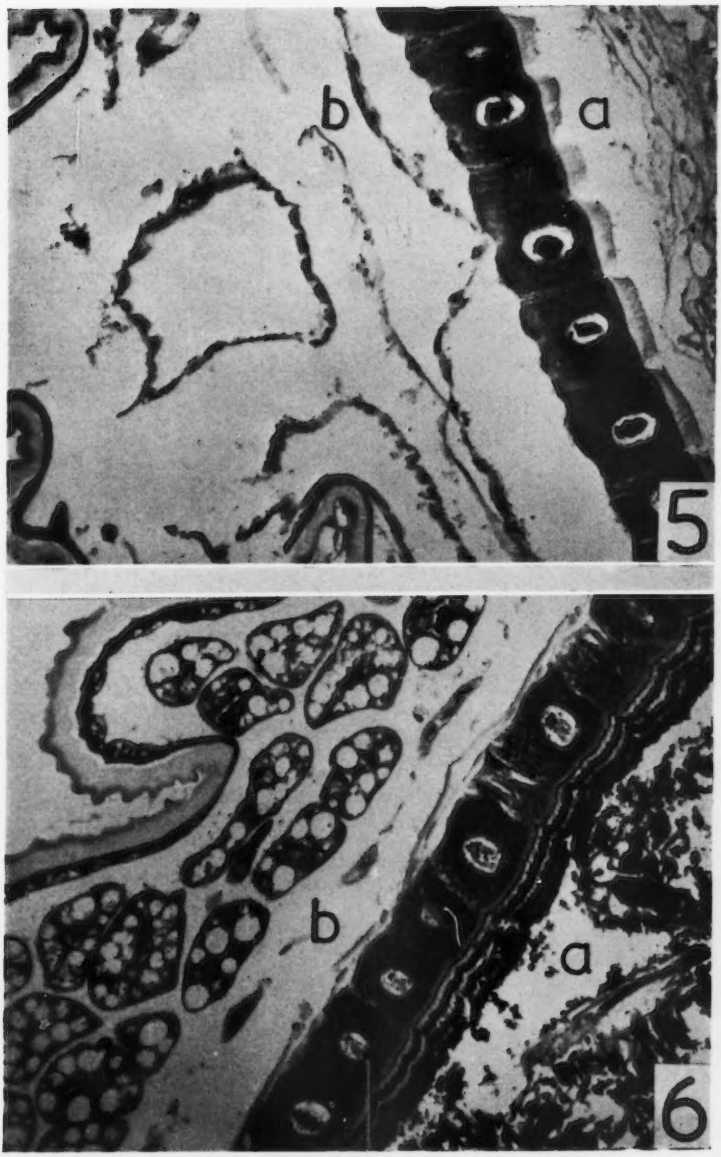


FIGS. 1 and 2. Sagittal sections of nerve cords of healthy and infected larch sawfly larvae.

FIG. 1. An abdominal nerve ganglion from a comatose larva. FIG. 2. An abdominal nerve ganglion from a healthy larva. Both 1400 ×.



FIGS. 3 and 4. Salivary gland sections from healthy and infected larch sawfly larvae. FIG. 3. Salivary gland tissue from a comatose larva. FIG. 4. Normal salivary gland tissue from a healthy larva. Both 900×.



FIGS. 5 and 6. Sagittal sections of midguts of healthy and infected larch sawfly larvae.

FIG. 5. Midgut epithelial cells from a moribund larva: (*a*) midgut lumen, (*b*) haemocoele, 350×. FIG. 6. Midgut epithelial cells from a healthy larva: (*a*) midgut lumen, (*b*) haemocoele. 400×.

PLATE IV

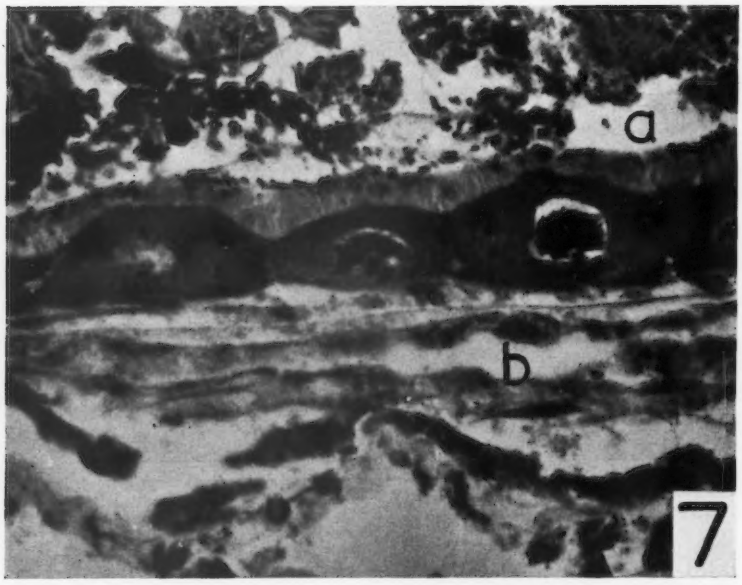
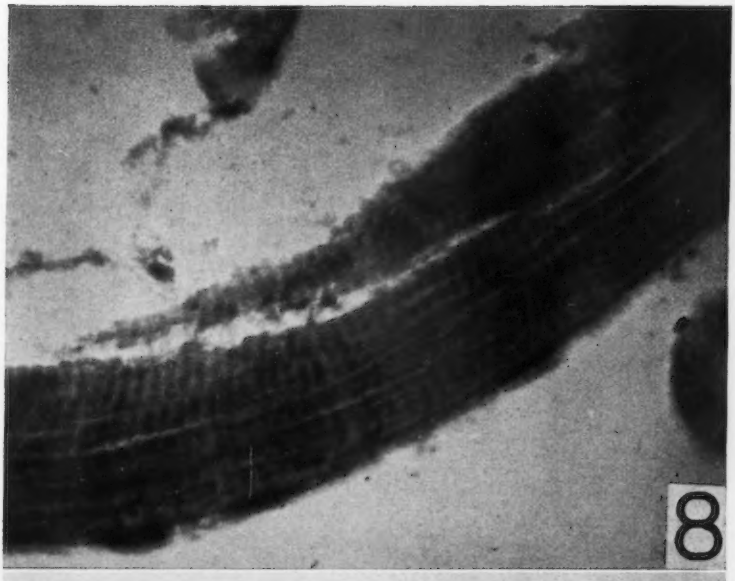
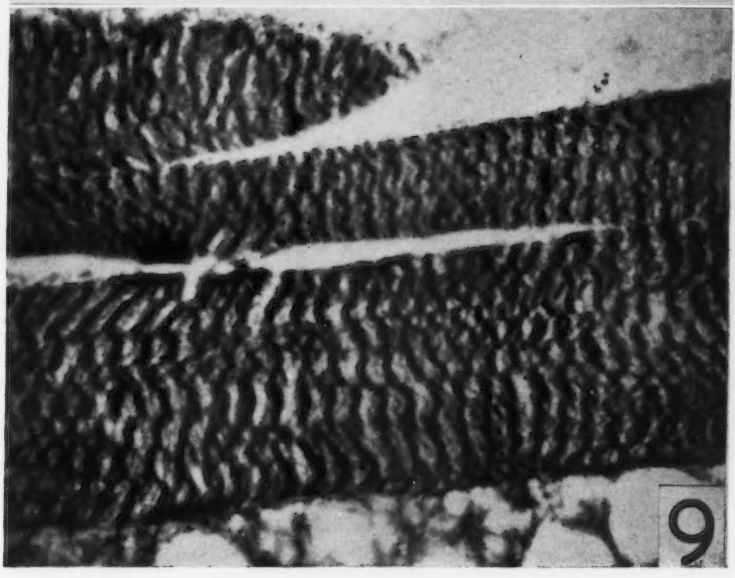


FIG. 7. Midgut epithelial cells from a comatose larch sawfly larva: (*a*) midgut lumen, (*b*) haemocoele. 1200×.





FIGS. 8 and 9. Sections of muscles from healthy and diseased larch sawfly larvae. FIG. 8. Muscle tissue from the haemocoele in a comatose larva. FIG. 9. Normal muscle tissue from the haemocoele in a healthy larva. Both 1150 ×.

Mouse Injections

The mouse injections were necessary to standardize the enzyme preparation made here, so that the larch sawfly LD_{50} might readily be duplicated.

Five-week-old mice of the Balb albino strain were injected intravenously with enzyme solution prepared from Lot VI. Preparations made from *B. megaterium* cultures were injected as controls. The mouse LD_{50} of Lot VI preparation was 0.048 mgm. The *B. megaterium* control preparation was non-toxic.

General Discussion and Conclusions

Were it postulated that in larch sawfly larvae infected with *B. cereus* lecithinase is the invasive agent, then two demands would have to be met.

1. Bacteria that do not produce lecithinase could not be pathogenic for the sawfly.

2. Strains of *B. cereus* that produce the highest amount of lecithinase should be the most virulent, and vice versa.

Several species of the genus *Bacillus*, including *B. subtilis*, *B. megaterium*, *B. laterosporus*, *B. para-alvei*, and *B. pumilis* do not produce lecithinase. These same species show no virulence for the larch sawfly. This portion of the preceding suppositions is relatively easy to show since the results can be only positive or negative.

The second stipulation is more difficult to test. In the first place the insect is parthenogenetic. It is probable that existing populations comprise clones that are genetically distinct. Secondly, variation of the bacteria in apparent virulence and ability to produce lecithinase may be expected. The pathogenicity tests conducted here establish that there are differences in ability of strains of *B. cereus* to kill the larch sawfly. Strain Mu-3055 proved to be the most virulent and Pr-1011 was the least. Moreover as demonstrated by the quantitative lecithinase tests, Mu-3055 produces the most lecithinase and Pr-1011 the least.

An analysis indicates that the correlation between lecithinase production and mortality caused by the bacterium in the 1952 tests is highly significant (see Table VIII); the correlation between the 1953 mortality data and lecithinase production is significant at the 5% level (see Table IX).

TABLE VIII

REGRESSION ANALYSIS OF 1952 FEEDING TESTS PLOTTED AGAINST THE LECITHINASE PRODUCTION OF SEVERAL STRAINS OF *Bacillus cereus*

Source of variation	Sums of squares	D.F.	Variances	*F* ratio	*P* value
Due to regression	7179.82	1	7179.82	15.47	<.01
About regression	9749.57	21	464.27		
Total	16,929.39	22			

TABLE IX

REGRESSION ANALYSIS OF 1953 FEEDING TESTS PLOTTED AGAINST THE LECITHINASE PRODUCTION RATIO OF SEVERAL STRAINS OF *Bacillus cereus*

Source of variation	Sums of squares	D.F.	Variances	*F* ratio	*P* value
Due to regression	163.64	1	163.64	5.23	<.05
About regression	938.83	30	31.29		
Total	1102.47	31			

The reason for the lower significance in the latter analysis is the atypical behavior of the two strains Mu-3013 and Ma-1041. These strains apparently are variable in lecithinase production and their pathogenicity cannot be measured accurately by this means.

This correlation, by itself, would be fairly convincing evidence, but when coupled with the fact that non-lecithinase-producing species of the genus *Bacillus* are not pathogenic for the larch sawfly, the logical conclusion is that lecithinase plays a major role in the invasion and destruction of larch sawfly larvae infected with high-lecithinase-producing strains of *B. cereus*.

These arguments are further strengthened by the observations that the pH of the anterior two-thirds of the midgut and of the blood of the larch sawfly lies within the pH range of lecithinase activity (15). In this connection it has been demonstrated that the sawfly pathogen Pr-1017 is not pathogenic, per os, for the tent caterpillar. The pH of the tent caterpillar midgut lies between pH 9.3 and 10.4 and Pr-1017 grows very slowly at pH 9.0 to 9.5; there could be no lecithinase production without multiplication of the bacteria. However Pr-1017 kills the tent caterpillar when injected into the body cavity; the pH of the tent caterpillar blood is approximately 6.7 and rapid multiplication of the bacterium occurs. It is significant that the pH 6.7 lies within the pH range for lecithinase activity.

There are some strains of *B. cereus* that are pathogenic for Lepidoptera possessing an alkaline midgut. *Bacillus sotto* (Ishiwata) (2) and *Bacillus cereus* var. *alesti* Toumanoff (28) are pathogenic for the silkworm, *B. mori*. *B. thuringiensis* is pathogenic for *Anisota rubicunda* (F.) and *B. mori* when fed in massive doses (Angus, personal communication).

Recently Hannay (11) showed that crystals are formed in sporulating cells of *B. thuringiensis*. According to Hannay, crystals were not found, in "... *B. cereus* strains not known to be pathogenic ..." for insects. These latter strains were not proved as non-pathogenic for insects, and it has been shown that several strains of *B. cereus*, pathogenic for the codling moth (26) and for the larch sawfly, do not produce these crystals. Studies conducted by Angus (1) strongly suggest that the crystal is connected with the virulence of the crystal-bearing strains of *B. cereus*, which are pathogenic for insects with a

relatively high alkalinity in the midgut (9.3 to 10.4) (2). The fact that Pr-1017 does not produce a crystal and grows slowly in the alkaline environment might be explanation for the lack of virulence of the sawfly pathogen for the tent caterpillar. It is significant that *B. thuringiensis* ranks third in order of pathogenicity for the larch sawfly; this rules out the possibility that its virulence for this insect is dependent on the presence of the crystal.

From the histological evidence it is clear that the action of the bacteria is primarily a toxic one. The fact that the bacteria break into the body cavity is of no immediate consequence, since the midgut cells and tissues in the haemocoele already show degenerative changes before the bacteria enter the blood. This would suggest that a soluble toxic substance (or substances) had been released by the bacteria and diffused into the blood prior to the entry of the bacteria. The nuclei and cell membranes contain relatively high concentrations of lecithin along with the lecithoprotein complexes usually found also in the mitochondria. The nuclei of the gut cells were first to show changes through the appearance of basophilic granules in the cytoplasm. There was no extensive proteolysis during the process of infection nor was there any sign of lipase activity, since the fat cells were often relatively unchanged in very sick larvae. Generally the histopathological picture is consistent with what would be expected were lecithinase the primary agent of virulence.

Other investigators have been interested in the connection between the production of phospholipase by strains of *B. cereus* and the pathogenicity of these strains for insects. S. E. Jacobs (personal communication) made preliminary investigations of the nature of the pathogenicity of strains of *B. cereus* for the meal moth, *E. kuehniella*, and considered the possibility of lecithinase as the virulent or invasive agent in preliminary studies. Toumanoff (29) suggested that lecithinase might be responsible, in part, for the pathogenic action of *B. cereus* var. *alesti* on the silk worm, *B. mori*.

The data presented in this paper strongly suggest that lecithinase is the virulent agent of *B. cereus* pathogenic for the larch sawfly. Further studies to test this theory are now being conducted along two main lines. An investigation of the effect of *B. cereus* infection upon the phospholipids in the host insect is under way. In conjunction with this work the effect of the enzyme, phospholipase C, upon the metabolism of the host is being studied, with special reference to those metabolic systems in which phospholipids play an essential role.

Acknowledgments

I wish to thank Drs. R. M. Belyea and S. G. Smith of the Forest Insect Laboratory and Dr. J. M. Cameron of the Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, for their advice and for their time in reading this manuscript. The photography presented here was done by Mr. D. C. Anderson, whose work is gratefully acknowledged.

References

1. ANGUS, T. A. A bacterial toxin paralysing silkworm larvae. Nature, 173 : 545. 1954
2. ANGUS, T. A. Some properties of a bacterial toxin affecting insect larvae. Can. Dept. Agr. Div. Forest Biol. Bi-mon. Progr. Rept. 10, (4). 1954.
3. BIRD, F. T. The use of a virus disease in the biological control of the European pine sawfly *Neodiprion sertifer* (Geoffr.). Can. Entomologist, 85 : 437-446. 1953.
4. BLOOR, W. R. Biochemistry of the fatty acids and their compounds, the lipids. Reinhold Publishing Corporation, New York. 1943.
5. BOND, J. H. and COLMER, A. R. A study of the lecithinase activity of *Bacillus cereus*. Proc. Louisiana Acad. Sci. 13 : 19-29. 1950.
6. BREED, R. S., MURRAY, E. G. D., and HITCHENS, A. P. Bergey's manual of determinative bacteriology. 6th ed. The Williams & Wilkins Company, Baltimore. 1948.
7. CHU, H. P. The lecithinase of *Bacillus cereus* and its comparison with *Clostridium welchii* alpha toxin. J. Gen. Microbiol. 3 : 255-273. 1949.
8. COLMER, A. R. The action of *Bacillus cereus* and related species on the lecithin complex of egg yolk. J. Bacteriol. 55 : 777-785. 1948.
9. FINKEL, A. J. The lipid composition of *Tenebrio molitor* larvae. Physiol. Zoöl. 21 : 111-113. 1948.
10. FRAENKEL, G. and HOPF, H. S. The physiological action of abnormally high temperatures of poikilothermic animals. (1) Temperature adaption and the degree of saturation of the phosphatides. Biochem. J. 34 : 1085-1092. 1940.
11. HANNAY, C. L. Crystaline inclusion in aerobic spore-forming bacteria. Nature, 172 : 1004. 1953.
12. HEIMPEL, A. M. Investigations of pathogens of the larch sawfly. *In* Ann. Tech. Rept. Forest Insect Lab., Sault Ste. Marie, Ont. 1952.
13. HEIMPEL, A. M. A strain of *Bacillus cereus* Fr. and Fr. pathogenic for the larch sawfly, *Pristiphora erichsonii* (Htg.). Can. Entomologist, 86 : 73-77. 1954.
14. HEIMPEL, A. M. An apparatus for mounting and holding insects. Can. Entomologist, 86 : 470. 1954.
15. HEIMPEL, A. M. The pH in the gut and blood of the larch sawfly, *Pristiphora erichsonii* (Htg.), and other insects with reference to the pathogenicity of *Bacillus cereus* Fr. and Fr. Can. J. Zool. 33 : 99-106. 1955.
16. JACOBS, S. E. Bacteriological control of the flour moth *Ephestia kuehniella* Z. Proc. Soc. Appl. Bacteriol. 13 : 83-91. 1950.
17. MCGAUGHEY, C. A. and CHU, H. P. The egg yolk reaction of aerobic sporing bacilli. J. Gen. Microbiol. 2 : 334-340. 1948.
18. METALNIKOV, S. and CHORINE, V. Experiments on the use of bacteria to destroy the corn borer. Intern. Corn Borer Invest. Sci. Repts. 2 : 54-59. 1929.
19. METALNIKOV, S. and CHORINE, V. On the infection of the gypsy moth and certain other insects with *Bacterium thuringiensis*. A preliminary report. Intern. Corn Borer Invest. Sci. Repts. 2 : 60-61. 1929.
20. PHILLIPS, C. M., BUCHER, G. E., and STEPHENS, J. M. Note on the preliminary field trials of a bacterium to control the codling moth. Can. Entomologist, 85 : 8. 1953.
21. REED, G. B. and MERKERCHER, D. C. Surface growth of bacteria on cellophane. Can. J. Research, E, 26 : 330-332. 1948.
22. ROEDER, K. D. Insect physiology. John Wiley & Sons, Inc., New York. 1953.
23. SMITH, S. G. Techniques for the study of insect chromosomes. Can. Entomologist, 75 : 22-34. 1943.
24. STEINHAUS, E. A. Principles of insect pathology. McGraw-Hill Book Company, Inc., New York. 1949.
25. STEINHAUS, E. A. Possible use of *Bacillus thuringiensis* Berliner as an aid in the biological control of the alfalfa caterpillar. Hilgardia, 20 : 359-381. 1951.
26. STEPHENS, J. M. Disease in codling moth larvae produced by several strains of *Bacillus cereus*. Can. J. Zool. 30 : 30-40. 1952.
27. TICHOMIROFF, A. Chemische studien ueber die entwicklung der insekteneier. Z. Physik Chem. 9 : 518-532. 1885.
28. TOUMANOFF, C. and VAGO, C. L'agent pathogène de la flacherie des vers à soie endémique dans la région des cevennes *Bacillus cereus* var. *alesti* var. nov. Compt. rend. 233 : 1504-1506. 1951.
29. TOUMANOFF, C. Description de quelques souches entomophytes de *Bacillus cereus* Frank. et Frank., avec remarques sur leur action et celle d'autres bacilles sur le jaune d'œuf. Ann. inst. Pasteur, 85 : 90-98. 1953.

MARINE BORER STUDIES: EVALUATION OF TOXICANTS[1]

By P. C. Trussell, C. O. Fulton, C. J. Cameron, and B. A. Greer

Abstract

Of 115 compounds tested for toxicity to adult *Bankia setacea* imbedded in wooden blocks, the most toxic was sodium arsenite, which gave a complete kill at 25 p.p.m. (as arsenic trioxide) after 18 hr. exposure. The relative toxicity of chemically pure sodium arsenite and various commercial concentrates to *Bankia setacea* was measured. The minimum concentration of sodium arsenite necessary to completely kill adult *Bankia setacea* was determined for exposure periods ranging from 10 min. to 24 hr. Limited observations were made on the toxicity of sodium arsenite to *Limnoria lignorum* and *Limnoria tripunctata*.

Introduction

The widespread damage to marine timbered structures and saw logs on the Pacific Coast caused by the marine borer *Bankia setacea* Tryon represents a serious economic problem. This study was undertaken to find a chemical toxic to *Bankia* that might be used for protecting saw logs during transit in infested waters.

Description of Organisms

Marine borers of the family Teredinidae include both the genera *Teredo* and *Bankia* of which the predominant species found in waters off the west coast of Canada is *Bankia setacea*. Settlement studies have shown that the main breeding season for this species is during the fall and winter months when water temperatures are low (4). In some locations a secondary breeding season may occur during the spring, while in others breeding may be continuous throughout the year. Recent larval distribution studies suggest that breeding may be more continuous than previously supposed (12).

The life cycle of this species is very similar to that of members of the genus found on the Atlantic coast, and has been adequately described by Clench and Turner (7). A fully developed *Bankia* is shown in Fig. 1.

The larva of *Bankia setacea* exists in a free-swimming stage for about one month (12) before it settles on wood and changes into the familiar elongated borer. Once settled the borer does not move during its lifetime, but continues to grow and enlarge its burrow. The rate of growth is dependent upon the salinity and temperature of the water, the food supply, and the available space. Teredinidae derive their food supply partly from the plankton in the water and partly from the wood passing through their digestive systems (15). Nutrient material and oxygen are taken in and waste products excreted through the two siphons, which normally extend beyond the surface of the wood.

[1] *Manuscript received April 4, 1955.*
Contribution from the Division of Applied Biology, British Columbia Research Council, Vancouver, British Columbia.

The siphons are the only part of the borer visible once it has burrowed into the wood. When a *Bankia* is disturbed or encounters unfavorable conditions it withdraws these siphons, and plugs its burrow by means of two pallets located at its posterior end. These pallets can be seen in Fig. 1. The entrance to the burrow is blocked if the borer detects contaminants in the water and this natural protective mechanism renders most biological toxicants ineffective.

During the current investigation some tests were made with *Bankia gouldi* Bartsch, which is responsible for much timber damage in the warm waters of the Gulf of Mexico and along the Atlantic coast of North and South America. All borers of the genus *Bankia* are very similar and the species are identified mainly by structural variations of the pallets.

Widespread damage to wood on the Pacific northwest coast is caused not only by the teredine borer, *Bankia setacea*, but also by the crustacean borer, *Limnoria lignorum* (Rathke). This borer is a small animal, about 1/8 in. long, which burrows mainly on the surface of the wood. It takes its oxygen directly from adjacent water, so rarely penetrates more than 1/2 in. below the wood surface. *Limnoria* apparently derive their food from wood (9), although they are able to live without it for several weeks. They are usually confined in a burrow, but are free to move about on the surface of the wood or migrate from one structure to another. In Pacific coast waters they breed at all times of the year, so that at no season is submerged timber safe from attack.

During this investigation a few tests were made on a warm-water species, *Limnoria tripunctata* Menzies, which is able to bore into creosoted timber and threaten permanent as well as temporary structures.

Controlling Marine Borers

To protect exposed, marine timbers against borer damage, either the wood must be treated with a chemical toxic to the borers, or the borers must be killed shortly after their establishment in wood. Pressure impregnation with creosote has been the most successful chemical treatment of wood and is generally used to prevent attack on permanent installations. However, impregnation treatments are impractical for temporary wooden structures, untreated installations already in place, or for saw logs. Among treatments that have been used or suggested for non-permanent installations are mechanical shock (blasting), electric shock, sonic and ultrasonic vibrations, and poisoning with toxic materials applied to waters surrounding the infested surfaces.

Blasting with subsurface dynamite charges has been used in British Columbia waters for several years to kill adult *Bankia* in saw logs and marine pilings (11). In 1946, after a severe teredo attack of the Queen's Dock, Swansea, Wales, small dynamite charges were released near the dock pilings and most of the borers were killed (3). Kirkwood (8) has reported, however, that charges of blasting powder set off at varying distances from blocks containing live *Bankia* appeared to have no deleterious effect. The main

PLATE I

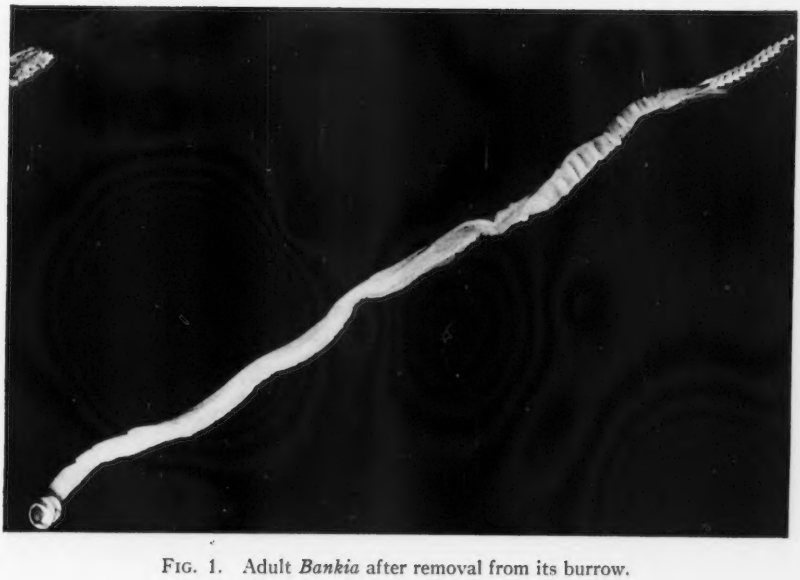


FIG. 1. Adult *Bankia* after removal from its burrow.

shortcomings of the blasting method are: difficulty of delivering shock waves to all the infested surfaces; the damage which sometimes occurs to the timbers being treated; and the killing of fish in the area. Blasting was therefore not considered a satisfactory method for protecting saw logs.

Reports on the effectiveness of electric shock and of chlorine produced by electrolysis of sea water are conflicting (6, 8, 10, 13). Experiments in these laboratories have shown that *Bankia* imbedded in wood are not adversely affected by electric shock or electrolytic chlorine. They withdraw their siphons on application of the current and extend them unharmed when the current is removed. The application of sonic or ultrasonic vibrations appears economically unattractive because of the high energy required for generation and transmission of the vibrations.

The use of chemical toxicants was considered the most practical experimental approach for protecting saw logs against borers. Various methods have been used to evaluate the effect of toxicants on borers (1, 2, 5, 14, 15, 16) and results have been dependent largely on the test method. For the present investigation several criteria were established for a toxicant for *Bankia* and *Limnoria:* (*a*) it must be lethal to the adult borers in their natural habitat (in wood); (*b*) it must be undetected by the borers; and (*c*) it must completely kill all borers within 24 hr. The results reported in the literature on the effect of chemicals on *Bankia* in wood have been disappointing. Allen and Carter (1) found that phenylarsenious oxide, mercuric oxide, and mercuric arsenate required seven days to kill imbedded *Bankia.* Kirkwood (8) reported that *Bankia* withdrew their siphons and plugged the openings to their burrows in the presence of copper and mercury salts. McQuaid (10) found that when chlorine was introduced into sea water, *Bankia* shut themselves off for days, and then continued their normal activities when placed again in uncontaminated sea water. Despite these discouraging results, the commercial benefits to be derived from an effective chemical toxicant warranted further investigation of this approach.

Materials and Methods

Test Organisms

Infested test blocks were obtained by immersing 2 in. cubes of Douglas fir in infested waters and allowing *Bankia setacea* to settle and grow in the blocks for three to five months. During the period of heaviest settlement in Vancouver harbor, which occurs between August and December, infested blocks were obtained locally. Otherwise, blocks were air-shipped from Massett Inlet, Queen Charlotte Islands, B.C., where *Bankia* settlement occurs more or less continuously throughout the year. These blocks were stored until required in the waters of Coal Harbour, Vancouver. Prior to use in the laboratory, infested blocks were immersed in fresh sea water for 15 min. and examined for *Bankia* siphons. If more than 10 pairs of siphons were present the blocks were considered suitable for toxicity tests. Since this investigation was concerned primarily with teredine borers, no special

efforts were made to procure specimens of *Limnoria lignorum*. However, many of the blocks also contained these organisms and, when organisms were present, the effect of toxicants on them was observed.

Similarly, specimens of *Bankia gouldi* and *Limnoria tripunctata* were obtained by immersing 2 in. cubic blocks of southern white pine in the ocean at Galveston, Texas. These infested blocks were air-shipped to this laboratory.

Preparation of Toxicant Solutions

Various concentrations of the toxicants in sea water (usually 200, 100, 50, 25, 12.5, and 6.2 p.p.m.) were prepared. Where possible, dilutions were made on the basis of the active ingredient. In the case of sodium arsenite solutions, the dilutions were based on the arsenic trioxide content. Toxicants of limited solubility in sea water were dissolved first in a small amount of organic solvent, previously tested for non-toxicity to *Bankia*, and then dispersed in sea water to the desired concentration. Details of the 115 chemicals examined are given in Tables I and II.*

Testing Procedures

Infested blocks were immersed in the toxicant solutions for specific intervals, usually 4 or 18 hr. During immersion the *Bankia* were observed for withdrawal of their siphons, which indicated sensitivity to the toxicant. After exposure the blocks were rinsed with fresh sea water, labelled, and immersed in the ocean for three to five days. They were then returned to the laboratory, placed in fresh sea water for one-half hour and examined for siphon activity. The results were recorded on the following basis: no active siphons—complete kill; one or two active siphons—partial kill; more than two active siphons—no kill. Adult *Bankia setacea* were tested with 115 possibly toxic compounds.

Results

The toxic effects observed for 18 compounds are listed in Table I. Of these compounds, only sodium arsenite gave a complete kill over a wide range of concentrations, from 12.5 to 200 p.p.m. (as arsenic trioxide) for 18 hr. exposure. Several other compounds gave partial kills over a range of concentrations, or gave complete kills at low concentrations where the *Bankia* apparently did not detect the toxicant. Dinitro-*o*-cyclohexylphenol gave a partial kill at 5 to 40 p.p.m., and octadecyltrimethylammonium salicylate gave a complete kill between 12.5 and 50 p.p.m., but not outside this range. Other special commercial chemicals were toxic at certain concentrations, but not through a wide range of concentrations.

** The manufacturers of the chemicals listed in Tables I, II, V, and VII are indicated by the following code letters: (A) American Cyanamid Co.; (B) California Spray-chemical Corp.; (C) Canadian Industries Ltd.; (D) Chipman Chemical Co. (Palo Alto); (E) Chipman Chemical Co. (Pasadena, Texas); (F) Chipman Chemical Co. (Winnipeg); (G) Dow Chemical Co.; (H) E. I. duPont de Nemours and Co.; (I) Emulsol Corp.; (J) B.F. Goodrich Co.; (K) Hercules Powder Co.; (L) Koppers Co.; (M) Monsanto Canada Ltd.; (N) Nuodex Products Co.; (O) Onyx Oil and Chemical Co.; (P) Pennsylvania Salt Manufacturing Co.; (Q) Rohm and Haas Co.; (R) Scientific Oil Compounding Co.; (S) Stauffer Chemical Co.; (T) Wyandotte Chemicals Corp.; (W) Imhausen Works Ltd. (Witten, Ruhr).*

TABLE I

CHEMICALS SHOWING TOXICITY TO ADULT *Bankia setacea* AFTER 18 HR. EXPOSURE

Trade name	Active chemical*	Manufacturer*	Toxic concentration, p.p.m.
	Complete kill, not detected		
	Sodium arsenite, C.P.		12.5 — 200
	Complete kill, but detected at higher concentrations		
Cr 1346	Capryldinitrophenyl acetate	Q	6.2 — 12.5
Er 2	Octadecyltrimethylammonium salicylate	Q	12.5 — 50
Er 109	—	Q	25
Er 162	Ethyl pyridine *p*-chlorobenzenesulphonate	Q	12.5
Lo 489	S-*t*-octyl-cresoxyethoxyethyl-N,N¹-dimethylthiouronium chloride	Q	50
Lo 566	Trichloromethyl-*p*-chlorobenzenethiolsulphonate	Q	25
Sm 314	Isobutyl benzoylacrylate	Q	100
Sm 529	—	Q	50 — 100
V 116	—	Q	25 — 50
	Partial kill		
Captan	N-(Trichloromethylthio)-4-cyclohexene-1,2-dicarboximide	S	25 — 200
Cr 1321	S-Benzyl thiouronium dinitrocaprylphenate	Q	100 — 200
Cr 1707	—	Q	200
DN No. 1	Dinitro-*o*-cyclohexylphenol	G	5 — 40
DN 289	Triethanolamine dinitro-*o*-sec-butylphenate	G	50 — 100
N 244	3-(*p*-Chlorophenyl)-5-methylrhodanine	S	200
—	Sodium-*o*-benzylchlorophenate	M	200
TEPP Spray concentrate	Tetraethyl pyrophosphate in xylene	M	200

* *See context.*

The chemicals listed in Table II showed no toxic effects on *Bankia setacea* at concentrations between 6.2 and 200 p.p.m. Nearly all these compounds caused the *Bankia* to seal off their burrows so that they were not directly exposed to the chemical. The few chemicals that were freely absorbed by the *Bankia* proved innocuous.

Comparison of Sodium Arsenite and Dinitro-o-cyclohexylphenol

The toxicities of sodium arsenite and dinitro-*o*-cyclohexylphenol are compared in Table III. As determined by a count of active siphons before and after treatment, an 85% kill of *Bankia setacea* was effected by a 24 hr. exposure to 5 p.p.m. dinitro-*o*-cyclohexylphenol, but increasing the toxicant concentration to 40 p.p.m. reduced the kill to 55%. Apparently the percentage of borers which detect this chemical increases with increasing concentration. *Bankia* are relatively insensitive to sodium arsenite since even at the 1% level (10,000 p.p.m. as arsenic trioxide) they do not retract their siphons. In this experiment, 12.5 p.p.m. of sodium arsenite (as arsenic trioxide) was not lethal to *Bankia setacea*.

TABLE II

CHEMICALS NOT SHOWING TOXICITY TO ADULT *Bankia setacea* AFTER 18 HR. EXPOSURE

Trade name	Active chemical*	Manufacturer**
	Inorganic chemicals	
	Antimony trichloride	
	Arsenious acid	
	Boric acid	
	Copper arsenite	
	Copper sulphate	
	Mercuric arsenate	
	Mercuric chloride	
	Potassium permanganate	
	Silver nitrate	
	Sodium arsenate	
	Sodium azide	
	Sodium fluoride	
	Sodium fluosilicate	
	Zinc arsenite	
	Zinc chloride	
	Zinc sulphate	
	Organic chemicals	
Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-1,4,5,8-dimethanonaphthalene	
Aerosol C-61	Ethanolated alkyl guanidine-amine complex	A
Aerosol SE	Stearamidopropyldimethyl-B-hydroxyethylammonium chloride	A
Ammonyx T	Cetyldimethylbenzylammonium chloride	O
Arex B.T.C.	Alkyldimethylbenzylammonium chloride	O
Chlordane	1,2,4,5,6,7,8,8-Octachloro-2,3,3*a*,4,7,7*a*-hexahydro-4,7-methanoindene	
Contact Herbicide	Pentachlorophenol	M
Copper 8	Copper naphthenate	N
—	Copper-3-phenylsalicylate	G
Cunilate 2174	Copper-8-quinolinolate	R
DDT	1,1,1-Trichloro-2,2-bis (*p*-chlorophenyl)ethane	
Dieldrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4*a*,5,6,7,8,8*a*-octahydro-1,4,5,8-dimethanonaphthalene	
—	Diphenylaminechlorarsine	L
DN No. 2	Dinitro-*o*-cresol	G
—	2,4-Dinitro-6-phenylphenol	G
Dowicide A	Sodium-*o*-phenylphenate	G
Dowicide G	Sodium pentachlorophenate	G
Dreft	Sodium lauryl sulphate	
Emcol C5-25	Ammonium salt of sulphated mono- and di-coconates	I
EPN	Ethyl-*p*-nitrophenyl thionobenzenephosphate	H
—	9-Fluorenone	
Gamma Hexane	Gamma isomer of benzene hexachloride	
Gamtox 1 Spray	Benzene hexachloride	B
—	2-Isopropyl-4,6-dinitrophenol	G
Methoxychlor	1,1,1,-Trichloro-2,2-bis (*p*-methoxyphenyl) ethane	C
—	Methyl alcohol	
Milmer No. 1	Copper-8-quinolinolate	M
N-168	3-Methyl-5-ethylrhodanine	S
N-521	3,5-Dimethyl-tetrahydro-1,3,5, 2H-thiadiazine-2-thione	S
N-536	3-*p*-Methoxyphenyl-5-methylrhodanine	S
N-551	2-(4-Chlorophenyl)-1,3-dithiolane	S
N-700	3-*p*-Tolyl-1,3-dithiolane	S
N-743	2-Ethyl-2-*p*-chlorophenyl-1,3-dithiolane	S
N-838	—	S
Oktone	Octochlorocyclohexanone	J

TABLE II *(Concl'd.)*

Trade name	Active chemical*	Manufacturer**
	Organic chemicals—(Cont'd.)	
Parathion	O,O-diethyl O-*p*-nitrophenyl phosphorothionate	C
p.e.p.s.	Polyethylene polysulphide	J
—	Phenol	
—	Phenylarsonic acid	
—	Phenylmercuric acetate	
Roccal	Alkyldimethylbenzylammonium chloride	
Rosin Amine D	Dehydroabietylamine	K
Rosin Amine D acetate	Dehydroabietylamine acetate	K
Rosin Amine D copper complex	Dehydroabietylamine copper complex	K
Rosin Amine D pentachlorophenate	Dehydroabietylamine pentachlorophenate	K
—	Rotenone	
Santobrite	Sodium pentachlorophenate	M
Sterichlor	Chloramine T	T
—	Strychnine	
—	Sulphite liquor	
Sulphenone	*p*-Chlorophenyl phenyl sulphone	S
Surf	Sodium alkyl aryl sulphonate	
—	2-*tert*-Butyl-4,6-dinitrophenol	G
Thanite	Fenchyl thiocyanoacetate	
Toxaphene	Chlorinated camphene	K
Vel	Ammonium monoglyceryl sulphate	
Witoxyl brown	—	W
Witoxyl pale	—	W
z.a.c.	Zinc dimethyl dithiocarbamate – cyclohexylamine complex	J
z.i.p.	Zinc dimethyl dithiocarbamate – cyclohexylamine complex plus polyethylene polysulphide	J
	Special commercial chemicals	
Cr 305	—	Q
Cr 353	Benzyldimethylphenylammonium-2,4-dinitro-6-methylphenoxide	Q
Cr 1294	Dinitrocaprylphenol	Q
Cr 1675	Sodium 4′-nitro,4-chlorobenzene sulphonanilide	Q
Cr 2536	5,5,7,7-Tetramethyloctenyl-2-cyclohexylamine	Q
Dithane Z-78	Zinc ethylene-bis-dithiocarbamate	Q
Er 25	*p*-Chloro-ω-nitrostyrene	Q
FW 129	Choline dinitrononylphenate	Q
FW 152	2,2-Dichloro-1,1-(*p*-chlorophenyl) ethanol	Q
He 761	—	Q
He 1054	Ethylenethiourea	Q
He 1142	ω-Nitrostyrene	Q
Hyamine 2389	Dodecylmethylbenzyltrimethylammonium chloride	Q
Hyamine 1622	Octylphenoxyethoxyethyldimethylbenzylammonium chloride	Q
Karathane	Dinitrocaprylphenyl crotonate	Q
Lo 77	2-(3′,4′-Dichlorobenzylmercapto)-imidazoline hydrochloride	Q
Lo 765	—	Q
O-2257	—	Q
O-2266	N-Cyanomethyl-(1,1,3,3-tetramethylbutyl) cyanamide	Q
Quarternary B-104	Didodecenyldimethylammonium chloride	Q
V 167	—	Q

* *Where available from supplier.*
** *See context.*

TABLE III

PERCENTAGE KILL OF ADULT *Bankia setacea* BY SODIUM ARSENITE AND DINITRO-*o*-CYCLOHEXYLPHENOL AFTER 24 HR. EXPOSURE

Sodium arsenite C.P.		Dinitro-*o*-cyclohexylphenol	
Concentration, p.p.m. As_2O_3	% kill	Concentration, p.p.m.	% kill
10,000	100		
1000	100		
100	100		
50	100		
		40	55
25	100		
		20	85
12.5	0		
		10	80
		5	85
		2.5	0

Results in Table IV show that the reactions of *Bankia gouldi* to commercial sodium arsenite solution and dinitro-*o*-cyclohexylphenol do not differ from those of *Bankia setacea*. In this instance, a concentration of 12.5 p.p.m. arsenic trioxide was toxic to both *B. gouldi* and *B. setacea*.

TABLE IV

COMPARATIVE TOXICITIES OF SODIUM ARSENITE AND DINITRO-*o*-CYCLOHEXYLPHENOL TO *Bankia gouldi* AND *Bankia setacea*

Chemical	Concentration, p.p.m.	Kill after 18 hr. exposure	
		B. gouldi	*B. setacea*
Sodium arsenite	50	Complete	Complete
Liquid concentrate	25	Complete	Complete
(Concentration as As_2O_3)	12.5	Complete	Complete
Dinitro-*o*-cyclohexylphenol	40	Partial	Partial
	20	Partial	Partial
	10	Partial	Partial

Evaluation of Commercial Sodium Arsenite Solutions as Toxicants

Several commercial sodium arsenite solutions were compared with "Chemically Pure" and "Technical Grade" sodium arsenite to determine whether commercial solutions would be suitable for large-scale use. Results listed in Table V show that with one exception the commercial preparations completely killed all *Bankia* in 18 hr. at 25 p.p.m. arsenic trioxide.

Using commercial liquid sodium arsenite, the minimum arsenic trioxide concentration lethal to *Bankia* was determined for varying exposure periods. Results are tabulated in Table VI. The lowest lethal concentration was 25 p.p.m. (as arsenic trioxide) and the shortest lethal exposure period was 10 min. at 1000 p.p.m. arsenic trioxide.

TABLE V

TOXICITY OF COMMERCIAL SODIUM ARSENITE PREPARATIONS TO *Bankia setacea*

Trade name	Chemical name	Manufacturer*	Min. lethal conc. p.p.m. As_2O_3	
			4 hr. exposure	18 hr. exposure
	Sodium arsenite (solid) C.P. $NaAsO_2$		25	12.5
	Sodium arsenite (solid) technical grade (75% As_2O_3)	F	—	25
	Sodium arsenite liquid concentrate (7.87 lb. As_2O_3 per Imp. gal.)	F	25	25
Atlas A-4	Sodium arsenite liquid concentrate (4 lb. As_2O_3 per U.S. gal.)	D	50	50
Atlas A-6	Sodium arsenite liquid concentrate (6 lb. As_2O_3 per U.S. gal.)	E	50	25
Penite 35	Sodium arsenite liquid concentrate (4.5 lb. As_2O_3 per U.S. gal.)	P	50	25
Penite 6X	Sodium arsenite liquid concentrate (9.5 lb. As_2O_3 per U.S. gal.)	P	25	25

* *See context.*

TABLE VI

MINIMUM CONCENTRATION OF ARSENIC TRIOXIDE LETHAL TO ADULT *Bankia setacea* AT VARYING EXPOSURE PERIODS USING COMMERCIAL SODIUM ARSENITE SOLUTION

Exposure period	Min. lethal As_2O_3 conc., p.p.m.
10 min.	1000
30 min.	1000
1.5 hr.	200
3 hr.	100
4 hr.	50
12 hr.	25

Tests on Limnoria Species

Samples of commercial liquid sodium arsenite concentrates were tested against two species of *Limnoria*, the local species *L. lignorum* and a warm-water species from the Gulf of Mexico, *L. tripunctata*. Results of limited tests are shown in Table VII. With both species the lethal concentration varied between 12.5 and 25 p.p.m. (as arsenic trioxide) for an 18 hr. exposure period.

TABLE VII

TOXICITY OF COMMERCIAL SODIUM ARSENITE PREPARATIONS TO *Limnoria* SPECIES AFTER 18 HR. EXPOSURE

Trade name	Chemical name	Manufacturer*	Min. lethal conc. p.p.m. As_2O_3	
			L. lignorum	*L. tripunctata*
	Sodium arsenite liquid concentrate (7.87 lb. As_2O_3 per Imp. gal.)	F	12.5	12.5
Atlas A-4	Sodium arsenite liquid concentrate (4 lb. As_2O_3 per U.S. gal.)	D	12.5	25
Atlas A-6	Sodium arsenite liquid concentrate (6 lb. As_2O_3 per U.S. gal.)	E	25	25
Penite 6X	Sodium arsenite liquid concentrate (9.5 lb. As_2O_3 per U.S. gal.)	P	—	25

* *See context.*

Discussion

Investigation on toxicity of chemicals has been intentionally limited to their action on *Bankia* imbedded in wood. Other methods of toxicity evaluation, for example those conducted on adult *Bankia* extracted from their burrows (1, 14) and on *Bankia* larvae (2, 5, 15), may lead to some practical applications, but do not necessarily indicate the effectiveness of poisons on imbedded borers. This is due to the ability of the imbedded borers to detect foreign chemicals and then protect themselves by withdrawing and sealing themselves inside their burrows. On the other hand, any poison killing adult *Bankia* by the methods reported here is of immediate potential commercial value. Of the 115 toxicants tested, a number were sensed by *Bankia*, which then sealed themselves in their burrows; others which were not sensed proved innocuous. The single exception was sodium arsenite, which was not detected by the borers, and in addition was lethal over a wide range of concentrations.

To be of practical value in treating marine timber, a chemical toxicant must kill all the borers present. If only a few remain after a treatment, these borers are able to grow larger than they otherwise would, and the ultimate damage is not appreciably lessened. Thus toxicants, such as dinitro-*o*-cyclohexylphenol, which give partial kills of *Bankia*, are of no value in protecting timber against damage by these borers. Chemicals which are toxic at low concentrations, but are detected by *Bankia* at higher levels, such as octadecyltrimethylammonium salicylate, must also be ruled out as useful toxicants for practical applications, since toxicants must be introduced at concentrations greatly in excess of their minimum toxic levels during a treatment. If the *Bankia* are sensitive to such concentrations, they will plug their burrows and, even if they open them after the chemical is diluted below the detectable range, the time of treatment would be materially increased. Therefore, of all the toxicants listed in Tables I and II, only sodium arsenite is of practical value.

Considering the ability of *Bankia* to sense other poisonous chemicals, their inability to sense sodium arsenite, even in concentrations as high as 1% (arsenic trioxide) is remarkable. No relationship between chemical composition and this inability to sense sodium arsenite was apparent, since even low concentrations of sodium arsenate caused the *Bankia* to seal off their burrows.

The killing of *Bankia* by sodium arsenite is a function of both concentration and exposure time, the greatest changes in this relationship occurring between the limits of 25 p.p.m. and 1000 p.p.m. for 12 and 0.5 hr., respectively. For an 18 hr. exposure the minimum lethal concentration varied between 12.5 and 25 p.p.m. arsenic trioxide.

When compared at the same arsenic trioxide level, differences were observed in the toxicities of commercial sodium arsenite preparations. For field studies, therefore, selection should be made on this basis.

Concentrations of sodium arsenite which are effective against *Bankia* have also proved effective against *Limnoria lignorum* and *L. tripunctata*. *Bankia* and *Limnoria* are frequently found together in nature, so both types of borers can therefore be controlled by the one chemical.

The advantages of sodium arsenite for killing marine borers may be summarized as follows: (1) it is highly toxic to both teredine and limnorial borers; (2) it does not cause teredine borers to close off their burrows; and (3) it is inexpensive enough to make feasible the treatment of large volumes of water surrounding infested wood. The chief disadvantage of sodium arsenite is its toxicity to a wide range of animal life other than marine borers.

Acknowledgments

The authors wish to express their appreciation to the Powell River Co. Ltd., for supplying infested blocks from Massett Inlet; to the Todd Shipyards Corp., Galveston, Texas, for supplying infested blocks from the Gulf of Mexico; and to the following firms for supplying samples of chemical toxicants: California Spray-chemical Corp., Canadian Industries Ltd., Chipman Chemical Co., The Dow Chemical Co., B.F. Goodrich Chemical Co., Hercules Powder Co., Monsanto Canada Ltd., Pennsylvania Salt Manufacturing Co., Rohm and Haas Co., Stauffer Chemical Co.

References

1. ALLEN, M. S. and CARTER, R. H. Toxicity of certain compounds on marine wood boring organisms, together with some physiological considerations. Marine Piling Investigations. Rept. Natl. Research Council. Washington, D.C. 1924.
2. BARGER, G. Experiments on the protection of timber against marine borers. Deterioration of structures in sea water. Dept. Sci. Ind. Research, pp. 11–23. 1935.
3. BELL, F. V. M. Maintenance of maritime structures. Notes on timber structures, with particular reference to maintenance of oil loading jetties at Queen's Dock, Swansea. Mimeographed. 1947.
4. BLACK, E. C. and ELSEY, G. R. Incidence of wood-borers in British Columbia waters. Fisheries Research Board Can. Bull. 80. 1948.
5. BRYAN, J. Deterioration of structures of timber, metal, and concrete exposed to the action of sea-water. 19th Rept. Comm. Inst. Civil Eng. (London). 1947.

6. CHELLIS, R. C. Finding and fighting marine borers. IV. Creosote impregnation and poisoning. Eng. News-Record, 140 : 344–347. 1948.
7. CLENCH, W. J. and TURNER, R. D. The genus *Bankia* in the western Atlantic. Johnsonia, 2 (No. 19). 1946.
8. KIRKWOOD, M. The teredo problem. The Powell River Co. Ltd., Vancouver, B.C. Tech. Rept. No. 50–134. Mimeographed. 1950.
9. KOFOID, C. A. and MILLER, R. C. *Limnoria* and its allies; the crustacean borers. Final Rept. San Francisco Bay Marine Piling Committee. San Francisco, Calif. pp. 306–338. 1927.
10. MCQUAID, M. S. The destruction of marine borers in piling by the action of chlorine generated by the electrolysis of sea water. Marine Piling Investigations. Rept. Natl. Research Council. Washington, D.C. 1924.
11. QUAYLE, D. B. The use of dynamite in teredo control. Progr. Repts. of the Pacific Coast Stas. Fisheries Research Board Can. No. 51, p. 20. 1942.
12. QUAYLE, D. B. The larva of *Bankia setacea* Tryon. Rept. B.C. Dept. Fisheries. pp. m88–m91. 1951.
13. RAMAGE, W. D. and BURD, J. S. Chemical investigations. Final Rept. San Francisco Bay Marine Piling Committee. San Francisco, Calif. p. 174. 1927.
14. SHACKELL, L. F. The comparative toxicity of coal tar creosote and creosote distillates and of individual constituents for the marine wood borer *Xylotryra*. Proc. Am. Wood-Preserver's Association, 11 : 233–252. 1915.
15. SMITH, F. G. W. Marine borer investigations. The Marine Laboratory, Univ. Miami. Final Rept. Office Naval Research Contract Nonr 705(00). 1954.
16. WAKEMAN, C. M. Discussion of recent laboratory biological survey methods for comparing various toxicants used to combat marine borers. Proc. Am. Wood-Preserver's Association, 43 : 220–221. 1947.

COMMONWEALTH BUREAU OF HORTICULTURE AND PLANTATION CROPS

EAST MALLING, KENT

The publications issued by this Branch of the Commonwealth Agricultural Bureaux include the following periodical and occasional publications:

HORTICULTURAL ABSTRACTS

A quarterly digest of world literature on research into the cultivation of horticultural and plantation crops including deciduous fruits, vegetables and ornamentals, drug plants, and tobacco.

Annual subscription 50*s* through a bookseller, or, within the Commonwealth, 40*s*. by direct subscription.

TECHNICAL COMMUNICATIONS

22. E. J. Hewitt. Sand and water culture methods used in the study of plant nutrition. 1952, pp. 241, bibl. 943, illus. 42*s*. "Here is a timely book which should be in the hands of all workers in the field of plant nutrition." Agric. Inst. Rev., 1953, Vol. 8, No. 5.

23. S. C. Pearce. Field experimentation with fruit trees and other perennial plants. 1953, pp. 141, bibl. 194. 10*s*. "It should be studied section by section by all engaged in experimental work on perennial crops, for it is full of wise advice." J. hort. Sci., 1953, Vol. 28, Pt. 4.

Subscriptions and enquiries to C.A.B., Farnham Royal, Slough, Bucks, England.

Free specimen copies of Horticultural Abstracts from Bureau, East Malling.

Notes to Contributors

Manuscripts

(i) ***General***

Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

Illustrations

(i) ***General***

All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used unless it is desired to have all the co-ordinate lines show. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil **(do NOT use typewriting),** and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. In large drawings or groups of drawings the ratio of height to width should conform to that of a journal page but the height should be adjusted to make allowance for the caption.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard with no space or only a **very** small space (less than 1 mm.) between them. In mounting, full use of the space available should be made (to reduce the number of cuts required) and the ratio of height to width should correspond to that of a journal page (4¾ × 7½ in.); however, allowance must be made for the captions. Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. An additional charge is made for illustrations that appear as coated inserts. The cost per page is given on the reprint requisition which accompanies the galley.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

Contents

Page

The Development of Sampling Techniques for Forest Insect Defoliators, with Particular Reference to the Spruce Budworm—*R. F. Morris* - - - **225**

Oogenesis in Several Free-living and Plant-parasitic Nematodes—*Roland H. Mulvey* - - - - - - - - - - - - - - - **295**

Investigations of the Mode of Action of Strains of *Bacillus cereus* Fr. and Fr. Pathogenic for the Larch Sawfly, *Pristiphora erichsonii* (Htg.)—*A. M. Heimpel* - - - - - - - - - - - - - - **311**

Marine Borer Studies: Evaluation of Toxicants—*P. C. Trussell, C. O. Fulton, C. J. Cameron, and B. A. Greer* - - - - - - - - - - **327**